Vitamin D-Volume 1 #102 7/19/74

VITAMIN D #102

E33

VOLUME 1

GRAS MONOGRAPH SERIES

VITAMIN D

prepared for
THE FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION
AND WELFARE

JULY 19, 1974

This publication was prepared under Contract Number FDA 72-100
with the Public Health Service, Food and Drug Administration,
Department of Health, Education, and Welfare

prepared by
Tracer Jitco, Inc.

Table of Contents

SUMMARY

Preface and Chemical Information

Vitamin D is not a specific substance, but a biological activity shown by a range of natural and synthetic compounds, in most cases after metabolism by the body. The bioassayed activity is itself an end result of several different biological actions, now recognised as hormonal actions.

Because knowledge of the many vitamins D has advanced rapidly during the past few years, this monograph contains a brief chronology of salient advances to provide a context for the reports abstracted in the monograph. A glossary following the preface contains some abbreviations used frequently in the monograph. These abbreviations are also used in the summary.

Acute Toxicity

With vitamin D substances, lethal toxicity is not always immediate. Therefore the data are also reported as found in short-term toxicity studies.

Sahashi et al. (4990) determined the LD_{50} of i.p. D_2 sulfate in mice (20g) to be 2,500,000 IU/kg BW.

Harris et al. (2438) found that rats fed 50,000 USP units daily of either irradiated ergosterol or tuna liver oil died after 17-31 days of feeding.

The equivalent toxic doses (20-day median survival time) for rats (300-600 g) given D_2, D_3 or dihydrotachysterol by stomach tube were found by McChesney (3821) to be 3.60, 2.30 and 1.00 mg/kg/day respectively

Rabbits died five to seven days after s.c. injection of D_2, by Matsudo and Kato (3770), of 4,000,000 IU/kg; 100,000 IU/kg or 10,000 IU/kg daily for three consecutive days.

All adult female rabbits given i.m. injections of 2.5, 3.5 or 4.5 million units (total amount) of activated ergosterol in cottonseed oil by Friedman and Roberts (1947) died within 65 days of their first injection.

Taylor and Weld (5713) fed a young dog (4 kg, 3-4 mos.) 3 ml irradiated ergosterol in three days. The dog died less than 48 hours afterwards.

Steck et al. (5512) found that more than 20,000 units/kg/day of activated ergosterol or calciferol in corn oil given per os to adult dogs was fatal to 35 out of 43 animals.

Debré (1344) reported the deaths of two infants, one (20 mos. old) administered 11,300,000 units D_2 and the other (16 mos. old) administered 18,200,000 units.

Henkin et al. (0161, 2550) reported the death of a 54-year-old woman who received 100,000,000 IU D per os and i.m. over three and one-half months.

Short-Term Toxicity

Harris et al. (2438) compared the pathological effects of feeding rats large daily amounts of either irradiated ergosterol or tuna liver oil. Daily feedings of 50,000 units of either D source produced calcification in the kidney, stomach, aorta, heart and lung. As measured by tissue pathology, the irradiated ergosterol was more toxic than the tuna liver oil.

McChesney (3821) found that in rats the toxicity of D_2 and D_3 apparently was correlated with the hypercalcemic effect, while the toxicity of dihydrotachysterol was greater than its hypercalcemic effect.

Constantinides (1126) fed rats a lipemia-producting diet followed by viosterol administration and reported the production of true intimal foam cell lesions in their arteries, particularly the coronaries. Fraser et al. (1896) produced predominantly calcium hydrogen phosphate kidney stones in rats by weekly feeding of 10,000 IU of D_3 for 45 days.

Coleman (1083) produced aortic lesions in rabbits by giving them, D_2 40-420 IU/g BW, or D_3 100-770 IU/g BW, p.o. The serum Ca levels of the experimental animals remained high long after withdrawal of D.

Taylor and Wald (5713) gave young pups irradiated ergosterol p.o. at ten times the therapeutic dosage. A large number died of intussusception, which the authors concluded was due to a D-induced disturbance of the peristaltic mechanisms of the bowel.

Hendricks et al. (2547) administered oral D to young dogs fed diets with Ca levels similar to that recommended for infants. Some toxic symptoms were seen in all dogs receiving D at 10,000 IU/kg/day. A premature animal had the most severe symptoms. All of the overdosed dogs had elevated serum calcium levels. The authors commented that the toxic effect of a single massive dose of D was more severe than that of repeated, moderately excessive doses; that premature infants might be unusually sensitive to hypervitaminosis D; and that a diet high in Ca might aggravate any such toxicity.

Fell et al. (1766) produced diffuse lesions in the aorta of a one-year-old sheep after a single i.m. injection of 1,000,000 units D_3. Localized arteriosclerotic lesions were found in the heart and lungs. Packett and Coburn (4415) found that a diet supplemented with D_3 at 200 IU/lb of feed enhanced urolithiasis in yearling sheep.

Kent et al. (3104) reported the effect on a monkey colony of a diet supplying 162,000 USP units D per animal daily, plus Ca and P. Lesions (calcium and iron deposits) were found mostly in the kidneys but also in the lungs, heart and salivary glands. A month after withdrawal of the high D diet, the surviving animals appeared to be healthy.

Cogan et al. (1074) reported five cases of D poisoning in adult humans in which the dosage ranged from 100,000 to 500,000 units daily for two-and-a-half months to five years. There was evidence of renal insufficiency in all cases; all were hypercalcemic and had band keratopathy.

Debré (1344) reported 21 cases of D toxicity in children, two of them fatal. He concluded that renal and cerebral impairment were the two main dangers of D toxicity in children.

A wide variation in the susceptibility of patients to the various D drugs administered was noted by Howard and Meyer (2793). Fatigue, weight loss, anorexia and vomiting were the chief symptoms. Impairment of renal function, and degenerative lesions with calcification, such as band keratitis, were the main findings.

Hyde and Richmond (2832) found irreversible kidney damage in a 12-year old boy given daily oral doses of 100,000 to 150,000 USP units of D for about 5.5 years, to treat rheumatoid arthritis.

Chaplin et al. (0972) reviewed 111 cases of D toxicity in the literature. In seven of their own patients taking 50,000 to 300,000 IU daily for periods from three weeks to six years, they consistently found band keratitis and metastatic calcium cysts.

Vitamin D intoxication has been reported as a cause of mental symptoms in adults by Lehrer and Levitt (3453). Their patients improved mentally after D medication was stopped.

Scharfman and Propp (5061) concluded that anemia was almost always present with D excess after observing four patients who had taken 50,000 to 150,000 units daily for some years and who had treatment-resistant normocytic, normochromic anemia. All showed symptoms of D toxicity with some renal impairment.

Paunier et al. (4473) observed 14 patients with rickets treated with D dosages of 25,000 to 250,000 IU/day for long periods. Nine patients suffered single episodes of D toxicity. The authors concluded that long-term D therapy could be relatively safe if the increase of the serum calcium level was determined frequently and accurately. Migrin et al. (4269), however, found that despite constant surveillance, calcification resulting in irreversible renal changes occurred in 10 out of 11 cases of rickets treated with large D doses.

A syndrome first described in 1952, "idiopathic hypercalcemia of infancy with failure to thrive" has been associated with prolonged high intakes of D (0656). Findings included intelligence and hearing defects, high serum Ca, impaired kidney function and demineralization of the long bones.

Coleman (1083) reported finding electrocardiographic changes in patients with infantile hypercalcemia which he interpreted as showing left ventricular myocardial damage. He suggested that congenital endocardial fibroelastosis, and the myocardial lesion of fibrocystic disease of the pancreas, could both be related to D.

Taussig (5703) and Beuren et al. (0526) reported a cardiac syndrome associated with D hypercalcemia, identified as supravalvular aortic stenosis (SAS). A Commission of the German Pediatric Society (5153) concluded that there was a connection between SAS and the severe form of idiopathic hypercalcemia, and suggested the possibility of individual sensitivity to D.

Long-Term Toxicity

Robertson et al. (4854) studied the effect of a moderate overdosage of D on the growth rate and longevity of male white mice. They found no difference in body weights at death between the experimental and control groups, but the life spans of the experimental group were slightly shorter than those of the control group.

Bills and Wirick (0566) fed activated ergosterol to rats from infancy to old age in doses ranging from 100 times to 40,000 times greater than the minimum "antiricketic" dose (mrd). At the lowest dose no effect was observed, but starting with 4,000 times the mrd, injurious effects became evident.

Gillman (2101) pointed out that human arteries were most susceptible to metabolic injuries during the first two years of life and that such injuries were precursors of adult atherosclerosis.

Special Studies

Gillman and Gilbert (2099) gave rats oral doses of 25,000 IU recrystallized purified D_2 and found damage, with or without calcification, in the aortas, coronaries, and myocardium and in the aortic, pulmonary, mitral and tricuspid valves.

Ornoy et al. (4389) gave 4,000, 20,000, or 40,000 IU D_2 i.g. by intubation to pregnant rats and found an alteration in the mineral composition of fetal bone at the largest dose. The authors commented that D_2 might pass through the placental barrier. This was corroborated by Haddad et al. (2337) who injected pregnant rats with (H^3)-D_3 or (H^3)25-OH-D_3.They found that by 48 hours almost 20% of the injected label was in the fetuses.

A number of cancer studies were reported:

Jones et al. (2976) could not correlate the serum Ca levels (in a 5 to 16 mg/100 ml range) with incidence of tumor in rats given 60 mg D_3 s.c. followed by i.v. injection of Walker sarcoma cells.

Schmid (5108) reported producing tumors in the gall bladders of guinea pigs with both irradiated ergosterol and AT_{10}, a factor isolated from irradiated ergosterol. The experiments were considered by the author to be too preliminary for evaluation.

Touraine and Zureick (5820) reviewed French reports claiming that D was carcinogenic to humans on the basis of molecular structure and clinical observations; they concluded that the evidence did not justify the claims but warranted further study.

Teratogenic studies were carried out by Friedman and Mills (1950), using rabbits. They explored the relationship between exposure to excessive amounts of D during pregnancy, and the development of the craniofacial complex and abnormalities of dentition found in children with SAS. Pregnant animals were given i.m. D_2 doses totaling 750,000 units each. From dental, skull, and other abnormalities observed in the experimental group offspring, the authors concluded that the cranial, facial, and dental signs of SAS, as well as the aortic lesions, might be due to a derangement of D metabolism during pregnancy.

Friedman (1949), reviewing the literature on SAS, commented that the wide variations of D-sensitivity, among both species and individuals, were still unexplained. He suggested that SAS might become preventable if pregnancies sensitive to teratogenic effects of D and related sterols could be recognized in time, and he recommended research into the epidemiology, genetics, metabolism, and pathology of SAS.

Kares (3105) reported observations of hypervitaminosis D at a children's hospital in Russia. He concluded that even small overdoses of D could generate symptoms, and that children varied widely in sensitivity to D. He noted that more Ca was absorbed from cow's milk when D was administered.

Fraser et al. (1894) reviewed the two forms of infantile hypercalcemia, and noted that cardiovascular involvement and mental retardation were signs of the severe form. They pointed out that a return of serum Ca levels to normal could impede accurate diagnosis of advanced cases. Their suggested treatment included reduction of daily Ca intake, elimination of D from the diet, and avoidance of sunlight.

Chinone (1024) found that a small amount of D stimulated follicular and uterine growth, and accelerated sexual function. A large continuous overdose had the reverse effect resulting in genital atrophy and cessation of sexual function. This was supported by Freedman (1920), who found vaginal changes in women administered totals of 2,250,000 to 2,550,000 units of D over 15-17 days.

Biochemical Information Breakdown

Stability data are given in the Chemical Information section. The relative heat-stability of D in oils led to its original discovery as a factor separate from vitamin A; see historical section of the preface.

Absorption and Metabolism

D levels in human serum did not indicate how much was absorbed, for they did not rise in proportion with massive D intakes. When such intakes were halted, serum D levels fell rapidly at first, but after four months the half-life of residual excesses in the body was found to be 16-17 months, indicating prolonged storage (3607).

A recent human study (5384) using low doses of labeled D_3 indicated that the half-life of D_3 in serum was 12 hours, and it was replaced by 25-OH-D_3 with a serum half-life of 19.6 days, accounting for 92% of the label.

When labeled D_3 and 25-OH-D_3 were given to pregnant rats, both compounds were found to be readily transferred to the fetuses, but the capacity of the fetuses to metabolize these compounds was not measured (2337).

Recently Omdahl and DeLuca (4376) doubted whether either the liver or the fat depots were primary storage sites, pointing to major concentrations in skeleton and muscle; they concluded that D was accumulated by lipid-rich tissues and cell components throughout the body.

Metabolism

Vitamin D_3 (4376) given p.o. or by injection is metabolized in the liver to 25-OH-D_3, which is converted by kidney mitochondria to $1\alpha,25\text{-}(OH)_2\text{-}D_3$, the final active hormone, or to other metabolites including 24, 25- and $25,26\text{-}(OH)_2\text{-}D_3$ which are thought to be still-active degradation-products. The liver step is regulated by feedback. The kidney produces mainly $1\alpha,25\text{-}(OH)_2\text{-}D_3$ when Ca intake is low, and mainly $24,25\text{-}(OH)_2\text{-}D_3$ when Ca intake is high, but this step is also controlled by PTH output and P intake.

The liver step can be bypassed by giving 25-OH-D_3. The kidney step can be bypassed by giving the synthetic analog 1α-OH-D_3. Both steps can be bypassed by giving $1\alpha,25\text{-}(OH)_2\text{-}D_3$ and perhaps also by synthetic analogs such as 25-OH-dihydrotachysterol$_3$.

D_2 is metabolized similarly (4376).

Excretion

Bile is the major route but is also concerned in absorption. Up to 30% of test doses of D_3 appeared in bile in 24-48 hours, and about 2% in urine, all as metabolites that were not fully identified. D_2 is theorized to be degraded and excreted more rapidly than D_3 by chicks (4376).

Effects on Enzymes and Other Bio-Chemical Parameters

Endogenous or exogenous D_3, or exogenous D_2, normally keep the plasma Ca high enough for normal bone calcification (4376) by:

(1) Absorption of Ca (perhaps also P) from the gut,
(2) Mobilization of Ca from bone into plasma,
(3) Reabsorption of Ca and P by kidney tubules, and
(4) Re-deposition of Ca into bone.

D_3 itself is inactive, and its principal metabolites act as follows for Ca:

(1) 25-OH-D_3: absorption, nil; mobilization, possibly.
(2) 1α,25-$(OH)_2$-D_3: absorption, directly.
(3) 24,25-$(OH)_2$-D_3: absorption, slightly; mobilization, active.
(4) 25,26-$(OH)_2$-D_3: absorption, active; mobilization and calcification, almost nil.

These metabolites may also influence P absorption by the gut and P and Ca reabsorption by the kidneys. PTH is the tropic hormone for the hormone 1α,25-$(OH)_2$-D_3, for PTH controls its rate of production, and PTH output is stimulated by low blood Ca and suppressed by high blood Ca (4376). In some conditions low P_i concentration may replace PTH release (5684).

In vitro (4376) 1α,25-$(OH)_2$-D_3 is 200-1000 times more active, and more rapidly active, than 25-OH-D_3 for Ca transport across membranes, and is 100 times more active (and more rapidly active) for Ca mobilization from bone. In vivo the hormone was more active at physiologic doses.

the 25-OH-D_3 at high doses, but when given p.o. for USP bioassay this comparison did not hold, and the authors concluded after other studies that the hormone turned over quickly and should be injected (4376).

Specific uses in D-resistant disorders of Ca metabolism were being studied for the following compounds (4376): 25-OH-D_3, 1α,25-$(OH)_2$-D_3, 5,6-trans-D_3 and its 25-OH derivative, dihydrotachysterol$_3$ and its 25-OH derivative, and 1α-OH-D_3.

Such disorders of Ca metabolism included "vitamin D-dependent" rickets in infants with adequate D intakes but with low serum Ca and P, high serum APase, and excessive urinary amino acids. This type of rickets responded to D_3 at 50,000 IU/day or more, or to 25-OH-D_3 at above 16,000 IU/day, or very rapidly to 1,25-$(OH)_2$-D_3 at 40 IU/day. Thus the authors pinpointed a recessive hereditary defect in the kidney enzyme 25-OH-D_3-1-hydroxylase as the cause of this type of rickets (1898).

In the D-resistant form of rickets defined as "X-linked dominant hypophosphatemia", excessive renal excretion of P_i was corrected by i.v. infusions of Ca, or by repalcement of P_i. Ca absorption was defective, and PTH levels were excessive. After various tests, the authors concluded that the impaired D metabolism was secondary to a genetic defect in the P_i transport systems of kidney and other tissues (2122).

Normal absorption of D_3 was found in a group of young adult patients with bone demineralization secondary to chronic kidney failure. In this case the mechanism of Ca BP synthesis was considered defective (0263), but its dependence on 1α,25-$(OH)_2$-D_3 remains uncertain (4376).

Although low blood levels of APase have long been diagnosed and more recently have been studied in relation to impaired osteogenesis and Ca absorption the question of whether the APase deficiency syndrome involves D or its metabolites is not yet settled (4376).

On the other hand, the cardiovascular system was clinically involved in the soft tissue calcification of hypercalcemic infants considered to be unusually sensitive to supplements of D (5218); and when cardiovascular damage was induced in rats by large excesses of D_3 added to their diets, alterations of APase activity in the aorta were among the major enzyme effects. (6353).

D_2 has been reported to improve the microscopical organization of osteogenesis independently of the amount of mineralization (2414).

Various effects of D have been reported on the metabolism of other minerals such as Mg (5219), Zn (0429), Cu (2283), and Mn (1158 , and upon the phosphorylation of thiamin (4691).

D has also been reported to have estrogenic activity (4382).

Drug Interactions

Diet supplements other than Ca and P can modify the response to D and vice versa. Toxic effects of excess D in rats were diminished when excess A was also fed (1056) but the literature is not unanimous on this. Lactose apparently can have a D-like effect in D-deficient rats when fed in place of starch (1595). Strontium in the diet has produced rickets in chicks by diverting the metabolism of 25-OH-D_3 from 1α,25-$(OH)_2$-D_3 to 24,25-$(OH)_2$-D_3 (4378). Diphosphonates may act similarly (4376).

Supplements of D can enhance or diminish responses to other steroids, depending on dosage and other factors. Synergism has been reported with cholesterol (1533). Both synergism and antagonism have been reported with estrogens (4382) and corticosteroids (4376), and the discrepancies remain to be resolved (4376).

A report that barbiturates accelerate the metabolism of D_3 has not yet been confirmed (4376).

Anticonvulsive drugs may produce hypocalcemia and rickets by causing the active metabolites of D to disappear, and definitive long-term studies are required (4376).

Consumer Exposure

Official Compendia

Neither the official compendia consulted, nor other searches, revealed any data or studies on quantitative exposures of the United States population to sunlight, or on UV irradiation of the land surface of the United States or of any region of the United States at any time of year. Thus, no base-line data were found for estimating the amounts of endogenous D activity generated naturally in the United States population.

Ingestible forms of D produced in the United States in 1969 totaled 10,000 lbs, or 177,805 billion IU; total sold was 4000 lbs, or 79,597 billion IU, with a value of $655 million, or $8.23 per billion IU (5878).

In 1971 the total of condensed and evaporated milks consumed in the United States was 1386 million lbs, or 6.8 lbs per capita, compared with the peak 20.4 lbs per capita reported for 1947 (6394).

For 1971 the total of margarine consumed in the United States was 2264 million lbs, or 11.1 lbs per capita, reflecting a continual increase from 5.8 lbs per capita reported for 1949 (6394).

In 1965 potential exposures to D were listed by the NAS NRC, in terms of fortification of the following foods (0080):

a. Prepared breakfast cereals, vitamin D-milk, evaporated milk, skim milk, infant dietary formula, Mellorine (vegetable-fat imitation ice-cream), margarine.
b. 250-1000 IU/lb in enriched flour (including bromated and self-raising flours), enriched cornmeal and grits, enriched macaroni and noodle products.
c. Enriched farina at 250 IU/lb, bread and rolls at 250-750 IU/lb, evaporated milk at 25 IU/fl. oz. of finished product.

Another source (0988) discussed some methods of adding D to foods. For example, D could be added to dry milk in a beadlet form, or by homogenizing in an oil carrier

before drying the milk. D_2 was stated to be the "common form used in human nutrition;" and because of analytical uncertainty in assaying low-potency products, "overages are necessary".

The 1972 FDMA Comprehensive GRAS Survey, based on a questionnaire, and published by the NAS NRC (@190) estimated some possible average daily intakes of D from the sources listed (based on total sample) and also maximal daily intakes (converted to μg):

	D_2		D_3	
	Average	Max	Average	Max
0-5 mo.	482	875	26	103
6-11 mo.	189	855	290	1388
12-23 mo.	160	384	257	814
2-65+ yr.	219	570	199	586

Carriers of D_2 listed were: baked goods, breakfast cereals other grains, fats and oils, milk products, meat products, poultry, sweet sauces, beverages, imitation dairy products, and baby foods. Carriers of D_3 listed were: fats and oils, milk products, and baby foods.

Information from Suppliers

Two major suppliers of milk to customers in the greater Washington (D.C.) area, each covering the entire area, responded to an invitation to state their policy and its results. It was known in advance that these were (1) a retail chain offering only vitamin D-milk, and (2) a milk producers' co-operative with 206 convenience stores offering both types of milk. Replies were as follows:

1. In 1969 both plain and vitamin D-milk were offered; by 1971 sales of plain milk had dropped, and its processing was no longer economic (@2092).
2. The policy was to offer the customer a choice (@903).

Total turnover for November 1973 was 1,029,117 gallons. Of this, 11% was homogenized milk with D, 59% the same without D; 3% was lowfat milk with D, 19% was lowfat milk without D; the remaining 8% was skim milks and cream, all with added D.

In 1973 a manufacturer (5895) stated, in response to an inquiry, that toxicological and clinical data were being developed with a view to making available 25-OH-D_3, 1,25-$(OH)_2$-D_3, and 1α-OH-D_3 as specific supplements for people with metabolic defects that currently resulted in their having to take massive amounts of D vitamins with all the attendant hazards.

Scientific and Survey Reports

In 1938 appeared the classic paper by Jeans and Stearns (2933) cited in most subsequent discussions of RDA (1849) in support of a level of 400 IU/day. However, the authors recommended not 400 but 300-400 IU/day, called this "tentative", restricted it to "only the ingested forms of vitamin D" known at that time, and cautioned that intakes considerably greater "may be detrimental", giving 1500 IU/day as an example. They further cautioned that the lowest harmful intakes had not been established. The authors emphasized that there was no basis for any recommendations for breast-fed babies, premature babies, older children, adolescents, or adults, including pregnant or lactating women. With these cautions, they provisionally suggested intakes ranging from 350 IU/day for children to 800 IU/day for pregnant or lactating women.

In 1966 Fraser et al. (1894) suggested that the Jeans and Stearns paper (2933) "does not stand up to modern tests of significance", and Taussig (5703) concluded that children susceptible to hypercalcemia had less than an 8-fold margin of safety above the RDA of 400 IU/day, recommending that physicians should avoid giving children doses that were unnecessary and might be harmful.

In 1968 Stearns (5504) concluded that 60-100 IU/day was enough to prevent rickets, that 300-400 IU/day was ample for maximal growth, and that growth was impeded above 2000 IU/day. She stated that many more babies in America were being overdosed than were being underdosed.

In 1970 Sealig (5217) concluded that D_2 was more toxic than D_3 to the renal and cardiovascular systems of experimental animals; that for human infants 95 IU/day in milk was adequate for prophylaxis; that even darkly pigmented children had been protected by 332 IU/day in milk, and such amounts could provoke hypercalcemia in fair-skinned children; that hypercalcemic damage to brain and arteries was usually irreversible at the time of diagnosis, whereas rickets could be diagnosed while damage was still reversible; and that the universal 400 IU/quart of milk was an "editorial compromise" and should be re-evaluated. The author noted that the potency of a given amount of D in milk was 3-10 times greater than in the carrier used for bioassay, which was oil.

In 1971 Lumb et al. (3607) surveyed some normal populations and found an average D level of 0.77 IU/ml of serum. They concluded that the normal or least-sufficient values were unknown, and the conventional 1 IU/ml was arbitrary; that there was more sunlight in North America than in Britain, and more sources of D were available; that adults probably required only 75 IU/day but casual exposure to sunlight was inadequate; and that adult Britons seldom ingested over 150 IU/day, but no reliable data existed for North America.

In 1973 Palmisano (4431) noted that most commercial milks, baby foods, and breakfast cereals were D-fortified; that average Americans could ingest several times the RDA; that amounts slightly over the RDA were profoundly toxic to some individuals (kidney calcification and hypercholesterolemia); that D-deficiency was rare except where malabsorbed; that the RDA was a maximum from all sources, and the minimum, 70 IU, was available in America from intermittent sunlight except perhaps in very dark-skinned people, and infants had less skin pigment than adults in all races; and that the new D_3 metabolites ought to lead to a new approach.

In 1973 a Lancet editorial (0193) noted that senior citizens received less UV and needed more supplemental D than younger adults. The Journal of the American Medical Association (1631) drew attention to the new D_3 metabolites.

Industrial Associations

In 1954 the American Institute of Baking (0079) advised its members not to add D to bread or, at any rate, not to add more than 400 IU/lb of flour and then to add Ca enough "to permit simultaneous claims for calcium". The Institute added that such addition "contributes little to the nutritional welfare of the American public".

In 1973 Brooks (0771) observed that response to the 1954 circular (above) was incomplete, members pointing out that many of the poor could not afford the extra penny that was then charged for D-milk.

Scientific Committees AAPCN

In 1963 the Committee on Nutrition of the American Academy of Pediatrics (AAPCN) (1118) issued a policy statement on D. They announced that:

1. 250 IU/day were at least as effective as greater intakes.
2. Negro infants did not need more than White infants.
3. Single doses of 300,000 IU were unnecessary and unphysiologic.
4. Premature infants should receive D in the first two weeks of life, but not more than 100-200 IU/day.
5. For older children and adults including pregnant or lactating women, needs could not be stated for lack of evidence.
6. Toxicity data were contradictory, but intakes far below 3000 IU could sometimes be toxic.
7. Exposures in the United States and Canada could reasonably exceed 3500 IU/day, with unknown long-term consequences. Only milk should be fortified.

The Committee also warned of the variety of D sources, reaffirmed an RDA for infants and children of 400 IU/day from all sources, and urged that commercial D supplements should be restricted to 400 IU/dose.

In further statements the AAPCN added:

1. Non-fat dried milk was not D-fortified because of a defect in the 1944 Standard of Identity; infants fed on it should receive D supplements (1116).
2. Infants should receive 400 IU/day, minimum 250, and formulas should contain 400 IU/100 kcal (1117).

AMACFN

In 1973 AMA Council on Foods and Nutrition (AMACFN) (1174) defined "enrichment" as addition of nutrients to conform with a Government standard; "restoration" as addition to replace losses in processing; "fortification" as addition of nutrients or quantities thereof that were never present. They (1848) endorsed all three procedures with regard to D in milk, fluid skim milk, and nonfat dry milk. In addition they recommended:

1. Standards should be adjusted for extra needs of identified groups in the population.
2. Enrichment or fortification should satisfy the following criteria:
   a. Enough people nutritionally deficient.
   b. Enough consumers of the proposed product.
   c. Bioavailability.
   d. The diet would not be unbalanced.
   e. No hazards of excess consumption.

NAS NRC

In 1968 the Food and Nutrition Board of the NAS NRC (1849) "reaffirmed" an RDA of 400 IU/day for D activity from all sources for infants, children and adolescents, adult males and females up to age 22, and pregnant and lactating women. They also advised night-workers and nuns to drink vitamin D-milk.

In support of these recommendations they stated:

1. D-deficiency arose when the total from UV exposure and ingested sources was inadequate.
2. Rickets had been prevented by 100 IU/day in normal and 200 IU/day in premature infants; it had been cured by 300 IU/day.

3. Beyond infancy, rickets was virtually unknown, and D requirements were hard to determine. The D requirements of adults were unknown.
4. Much less than 2000 IU/day were toxic to some people, and long-range effects of small excesses had not been studied "extensively".
5. Real intakes were hard to assess because so many foods were fortified. Excessive intakes were common, and most people of all ages received the RDA without supplements (except infants fed breast milk or unfortified formulas).

In 1973 the Board issued a policy statement (1848) intended to supersede the 1968 RDA statement although quantities of D were not mentioned:

1. All RDA were contained in a properly selected diet.
2. D should be added to milk, fluid skim milk, and nonfat dry milk.
3. Standards should be adjusted to extra needs of identified population groups.
4. A food should be "fortified" or "enriched" only when:
   a. Enough people ate it regularly.
   b. Enough were deficient of the added nutrient.
   c. The nutrient was stable and available.
   d. The diet would not be unbalanced.
   e. No hazards from heavy consumption of the food.
   f. The cost was reasonable.

WHO/FAO

In 1970 the report of a Joint WHO/FAO Expert Group (2969) stated that exposure data were still lacking, although they accepted an estimate of 116–133 IU/day for Britain.

They recommended 400 IU/day from birth to age 6, and for pregnant or lactating women, and 100 IU/day for others over age 6.

In a further 1970 report the WHO/FAO (1844) concluded that current programs of fortification prevented neither rickets nor hypervitaminosis D.

Regulatory Status - USA

The present United States law on the sale and labeling of products that are sources of D is contained in two FDA Regulations issued in 1973, which acquired legal force on January 1, 1974, with certain provisions effective on January 1, 1975 (0197,0198):

1. The Regulations cover all "foods" to which D has been added, and natural sources of D for which a claim is made on the label.
2. The "U.S. RDA" is 400 IU/day.
3. The label will specify any consumer group(s) for whom the product is specially intended:
   a. Infants
   b. Children under 4 years
   c. Older children and adults
   d. Pregnant or lactating women
4. If a natural source of D contains 10% or more of the U.S. RDA, it can be called a "significant source".
5. If a serving or dose-unit (e.g., pill) contains 50% or more of the U.S. RDA, after D has been added, the preparation must be described as a "dietary supplement". Exempt are natural sources of D, if additions no more than restore the natural D content.
6. If a single serving or dose-unit contains more than 100% of the U.S. RDA for vitamin D, it shall be a prescription-only drug. Exempt are foods for use "solely under medical supervision" by people with poor absorption of D; these may contain up to 1000 IU/dose-unit or recommended daily intake, and may be bought without prescription.
7. The Regulations do not otherwise restrict over-the-counter sales of D. Nor do the Regulations mention the GRAS List.

PREFACE

The title of this monograph is "Vitamin D" and its subject matter includes vitamins D, D_2, and D_3. The last two are chemically defined substances, like others in the GRAS Monograph Series. However, "vitamin D" is not a specific substance but a biological activity. Hence the need for a preface to this monograph.

Vitamin D activity is exhibited by many compounds, natural and synthetic. The natural ones occur widely, and not all have been identified. The specific activity is antirachitic, and it can be elicited by ultraviolet irradiation of the subject, the compounds, or materials containing the compounds. The activity is measured by bioassay, standardized on a dietary procedure for inducing and then curing rickets in rats, with a reference standard consisting of a measured amount of one compound, vitamin D_3 or cholecalciferol.

However, it has become known that the curing of rickets, as bioassayed, is an end result of several biological actions, that some compounds exhibit in proportions that differ from those exhibited by the reference standard. Some compounds discovered recently, or synthesized in the laboratory, have potencies that are high but selective and are not fully revealed by the official bioassay procedures.

Such potencies are currently under intensive study, for example, for specific low-dose treatments for disorders of calcium metabolism that hitherto have been treated with massive intakes of vitamin D compounds. Some of the new compounds are being considered for large scale manufacture.

Thus the entire field of "vitamin D" is currently in a phase of rapid change, due to advances in knowledge.

In addition, the term "vitamin" as applied to the vitamins D has been controversial for more than fifty years. Early researchers believed that the activity was hormonal. Then it was shown that factors promoting the activity could be ingested. The most recent research has established that some of the ingestible factors qualify to be called "prohormones" because they are metabolized to active substances that are hormones.

Thus conclusions reached in many of the reports abstracted in this monograph can be reinterpreted in the light of later discoveries. To provide context for readers who are not currently active in vitamin D research, a brief chronology of salient advances in knowledge will be found immediately after the glossary that follows this preface.

Glossary

A	vitamin A
AAP	American Academy of Pediatrics
AAPCN	Committee on Nutrition of the AAP
AMA	American Medical Association
AMAFCN	Council on Foods and Nutrition of the AMA
APase	alkaline phosphatase, phosphomonoesterase I
ATP	adenosine triphosphate (ADP is the diphosphate, AMP the monophosphate
ATPase	adenosine triphosphatase
BP	(Years) before the present
BUN	blood urea nitrogen
Ca	Calcium
CaBP	calcium-binding protein
cAMP	cyclic 3', 5'-adenosine monophosphate
CoA	coenzyme A
D, D_2, D_3	Vitamins D, D_2, D_3
DNA	deoxyribonucleic acid
ESR	erythrocyte sedimentation rate
FAO/WHO	Food & Agriculture Organization, World Health Organization of the United Nations
FDA	Food & Drug Administration of the United States
GRAS	Generally recognized as safe
HEW	United States Department of Health, Education, and Welfare
i.g.	intragastric(ally)
i.m.	intramuscular(ly)
i.p.	intraperitoneal(ly)
IR	infra-red radiation
IU	International Unit
i.v.	intravenous(ly)
mg%	milligrams per 100 milliliters
mos.	months
mp	melting point
mRNA	messenger ribonucleic acid
mw	molecular weight

NAD	nicotinamide adenine dinucleotide; NADP is its phosphate salt; NADH, NADPH are the reduced forms. Synonymous with the older terms DPN, TPN, DPNH, TPNH respectively.
NAS NRC	National Academy of Sciences, National Research Council
ng	nanograms, replacing the older term millimicrograms
nm	nanometer(s), replacing the older term millimicrons or millimicrometers
nmr	nuclear magnetic resonance
npn	non-protein nitrogen
$1,25-(OH)_2-D_2$	1,25-dihydroxyergocalciferol, the hormonal form of vitamin D_2.
$25-OH-D_2$	25-Hydroxyergocalciferol, or 25-hydroxyvitamin D_2
$1,25-(OH)_2-D_3$	1,25-dihydroxycholecalciferol, the hormonal form of vitamin D_3. (also found in the literature as DHCC).
$25-OH-D_3$	25-Hydroxycholecalciferol, or 25-hydroxyvitamin D_3 (also found in the literature as HCC).
P	Phosphorus
P_i	inorganic phosphorus (phosphate)
PGA, PGB, PGE	prostaglandins A, B, or E.
p.o.	per os, oral(ly)
PTH	parathyroid hormone, or extract of parathyroid gland used as a source of the hormone
rbc	red blood cell(s)
RDA	Recommended daily allowance(s)
RNA	ribonucleic acid
SAS	supravalvular aortic stenosis
s.c.	subcutaneous(ly)
sp, spp	species (singular, plural)
TCA cycle	tricarboxylic acid cycle, or Krebs cycle
tsp	teaspoonful
US, USA	United States of America
USP	United States Pharmacopoiea
UV	untraviolet radiation
vs.	versus
v/v	volume/volume
w/v	weight/volume

Degrees of temperature: centigrade (Celsius) unless otherwise specified.

CHRONOLOGY OF SALIENT ADVANCES IN KNOWLEDGE OF THE VITAMINS D

The outline history of the recognition of rickets, its etiology, the roles of vitamin D, and the final acceptance of the active metabolites of this vitamin as hormones, have been abstracted in part from a number of review papers (0837, 1429, 2887, 2577, 4212, 5204, 5218), and in part from the original documents.

Rickets was described in England about 1650 when air pollution first became severe, following the introduction of soft coal. In the early 19th century Wendelstadt described its occurrence at Wezlar, Germany (3577).

In 1824 Schutte recommended the use of fish liver oils, seemingly for night-blindness, since the functions of vitamins A and D were not recognized separately at that time (0837).

In 1854 Thompson wrote that the value of coconut oil was as great as that of cod liver oil, unlike almond or olive oils; and because coconuts were sun-dried before oil extraction, this has been claimed as the first specific recommendation of an irradiated oil (5204).

In 1864 Blake proposed that Neanderthal Man, claimed by some as a precursor of Homo sapiens, was simply H. sapiens with severe rickets (2887). In 1872 this opinion was supported in detailed arguments by Virchow. In 1970, after a century of disfavor, it was again argued by Ivanhoe (2887) on the grounds of:

a. Gross and detailed morphology, which could be verified by modern techniques, and in some cases had been verified;
b. Geographical distributions of finds;
c. Age of finds limited to period ca. 70,000-35,000 BP, coincident with overcast climate and cold that drove man into caves; attenuated symptoms associated with high altitudes or intervals of climatic remission;
d. Absence of evolutionary precursors; and
e. Absence of fishing equipment and paucity of fish remains at Neanderthal sites; signs of fishing began post-Neanderthal, about 30,000 years BP.

In 1884 Kassowitz observed that the incidence of rickets was seasonal, and attributed it to winters indoors (3577).

In 1888 Bland-Sutton documented rickets as endemic among animals at the London zoo but not at Manchester, Dublin, or other British zoos. In 1906 Hansemann called rickets a disease of "domestication" (3577).

In 1889 the British Medical Association prepared maps showing a relationship between rickets and cities. In 1890 Palm observed the absence of rickets in Japan and concluded, from correspondance, that rickets was confined to northern Europe and related to lack of sunlight (3577).

In 1909 Schmorl confirmed that the incidence of rickets was seasonal, by analysis of 386 autopsies on children younger than four. In 1912 Raczynski reared one puppy in sunlight and its littermate in the dark. The latter developed rickets, and its bones contained 36% less calcium than those of the puppy reared in sunlight (3577).

In 1912 Funk coined the word "vitamin" (0837).

In 1913 McCollum and Davis reported that an accessory food factor needed for growth was present in ether extracts of butter or egg, but not of lard or olive oil. In the same year Osborne and Mendel found that milk contained such a substance, that was present in the butter-fat, and in 1914 they demonstrated its presence in cod liver oil. Also in 1914, Drummond separated what is now called vitamin A from cholesterol, part-purified it, and concluded that it was an unsaturated sterol (0837).

In 1918 Findlay and Paton fed butter to puppies with rickets at Glasgow, and found that it increased the severity of rickets. At Bombay, Hutchinson observed an absence of rickets among poor Hindus who lived outdoors, and frequent rickets among well-fed upper-class Hindu and Moslem infants who were kept indoors; he cured ten of these by simply exposing them to sunlight. A study of 200 Glasgow families by Ferguson revealed that rickets was ten-fold more frequent among children kept indoors than among those who were allowed out. Thus rickets due to lack of sunlight appeared to be aggravated by rich feeding (3577).

In 1919 Huldshinsky at Berlin cured four cases of rickets in less than two months by exposure to artificial UV irradiation. He showed the involvement of a hormonal system by irradiating one arm and demonstrating, by x-rays, calcium deposition in the bones of the other arm (3577).

In 1919 Mellanby induced rickets in dogs, at London, by feeding them an unbalanced diet that he called "rachitogenic" (3577). In 1921 Sherman and Pappenheimer produced rickets in rats fed low-phosphorus diets (3577). Then in 1922 McCollum found that cod liver oil, when heated, lost its vitamin A activity but could still cure rickets. He named the antirachitic factor "vitamin D" (3577).

In 1921 Howland and Kramer reported that in rachitic persons the serum (Ca x P) level was too low to permit calcification of bone (4376). In 1923 Orr and co-workers in America reported high fecal Ca loss by patients with rickets, and suggested that vitamin D must act to increase Ca absorption, but the suggestion was not accepted by others (4376).

In 1923 Steenbock and coworkers at the University of Wisconsin showed that birds responded better to vitamin D_3 than to D_2 (4376).

In 1923 Park, also in America, reviewed the evidence and concluded that the underlying deficiency in rickets was endocrine, not dietary (3577).

However, in 1924, Steenbock showed that rickets could be prevented by irradiation of the diet (1426), and in 1925 Steenbock and Black (5513) reported that their non-irradiated Ration 2965 was better and more consistent than any other diet tested for producing experimental rickets in rats. Among other advantages, it did not produce signs of vitamin A deficiency.

Shown in Table 1, this diet became known as the Steenbock diet, and for half a century has been a standard method of producing experimental rickets in rats.

Table 1. The Steenbock Diet (5513)

Yellow corn	76%
Wheat gluten	20%
$CaCO_3$	3%
NaCl	1%

The authors (5513) also concluded that:

a. Too much irradiation inactivated olive or cod liver oil, but olive oil kept its potency when stored for about 10 months in the dark.

b. Crystallized cholesterol and its acetate and benzoate salts could be activated, and too much irradiation did not destroy their activity.

c. Mineral oil could not be activated.

d. The antirachitic factor of irradiated oils and fats was in their unsaponifiable fractions.

e. The unsaponifiable fractions could not be activated when the oils were aged, but only when they were fresh.

f. Acidity did not influence the capacity for activation.

In 1927 Windaus and Hess, and also Rosenheim and Webster, reported that ergosterol could be activated by irradiation (6048). In 1928 Windaus was awarded the Nobel Prize for studies on "the constitution of the sterols and

their connection with the vitamins". This effectively smothered the conclusion that Parks had reached in 1923 (3577).

In 1931 the League of Nations formally proposed the International Unit as the standard of D activity (4212).

In 1932 teams led by Windaus and by Askew isolated and identified vitamin D_2 (1429).

In 1933 Greaves and Schmidt demonstrated the need for bile salts in the absorption of vitamin D given by mouth (4376).

In 1934 Waddell studied the provitamins D, as the antirachitic factors were now called. He discovered that irradiation of "crude" cholesterol produced a different provitamin from that present in irradiated ergosterol: it was more active in chicks than in rats, and was inferred to be the main provitamin D of human tissues. Comparing milk with cod liver oil, Waddell concluded that the D factors in milk "may possess virtues still not understood but which may be explained for the moment on the assumption of better absorption" (6048).

In 1934 Barnes pointed to differences of potency among cod liver oil, viosterol, and milk, and to species differences of response among chicks, rats, and human infants. Thus bioassay data might not be clinically valid, and after studying 38 infants he recommended standardization of clinical procedures for evaluating different sources of D (0354). In the same year the USP unit was standardized to agree with the recommended International Unit to measure the activity of 0.025 μg of D_3 (4212).

Also in 1934 Dodds (1522) first reported estrogenic effects of ergosterol and calciferol when given in high doses to spayed rats.

In 1936 Lewis conducted a clinical study of the vitamin D responses of Negro, Hispanic, and White children in New York. He found that the Negro children were most liable to rickets and responded least to low-dose supplementation with D in milk (5218).

In 1936 Windaus reported the identification of vitamin D_3 with activated 7-dehydrocholesterol (4376). After confirmation by Schenk in 1937, it became assumed that D acted unaltered (4376).

In 1937 Nicolaysen reported that Ca absorption was increased by D (4376).

In 1938 Jeans and Stearns (2933) published a clinical report that became and remained the principal basis for the RDA, although the authors emphasized the lack of sound evidence and the need for caution. Their recommendation that infants receive 300-400 IU/day was "tentative"; the lowest

effective dose had not been established, and 1500 IU/day "may be detrimental". They noted that there was no evidence as to the dose-responses of children, adolescents, or adults.

However, in 1944 Johnston (2966) reported a clinical series from which he concluded that D elicited "no evidence of depressing effect" on growth when given to children as a concentrate in amounts ranging from 650 to 3,900 USP units/day.

In 1941 Harrison and Harrison reported that vitamin D increased the kidney tubular reabsorption of phosphate in dogs, but this report was doubted by others because the parathyroids were not removed (4376). In 1949 Migicovsky and Elmslie (3954) reported that in chicks, D given by mouth diminished the excretion of Ca but not of P, which was diminished by Ca ingestion.

In 1952 appeared the first reports of idiopathic hypercalcemia by Lightwood and by Fanconi and coworkers, describing signs typical of gross overdosage with vitamin D in infants who had not received supplements (6495).

As shown by Nicolaysen and Eeg-Larsen in 1953, it was then recognized that large amounts of D tended to decalcify bone, rather than to mineralize it. From 1952, Carlsson and coworkers proved this by tracer experiments (4376).

In 1955 Kodicek synthesized C^{14}-labeled D_2, and in 1956 he began to study the possibility that it was active only after metabolism, but the specific activity was too low for him to detect metabolites (4376).

In 1958 Fellers and Schwartz (1773) suspected that, in hypercalcemics, a sterol other than D_3 was responsible for D activity.

In 1958 Neuman and Neuman suggested that blood was normally supersaturated with Ca and P, and in the same year Neuman went on to show that in rickets the blood was less saturated with Ca and P than the bones. Thus they confirmed Howland and Kramer's report of 1921 (4376).

Also in 1958 Harrison, Harrison and Park showed that vitamin D was required to mobilize Ca from bone (4376). (This agrees with the concept that rachitic blood has less Ca than normal blood).

In 1959 Rasmussen first observed the role of PTH in Ca mobilization, working with isolated rat intestines (4376). He went on to study the role of calcitonin (5684).

Also in 1959, and using isolated rat intestines, Schachter and Rosen demonstrated that Ca was transported actively, against a concentration gradient, by an energy-requiring system of finite capacity (4376).

In 1964 Thompson and DeLuca (5772) reported another effect of D that was not Ca-dependent -- D_2 tripled the incorporation of P into phospholipids in the gut mucosa, somewhat less in the kidney, but not at all in the liver. They concluded that this would assist Ca transport if enough Ca were present.

In 1965 Wasserman and Taylor isolated and characterized a D_3-dependent specific Ca-binding protein that was part of the outer membrane of intestinal microvilli, but in 1973 its precise role in the sequence of Ca-transport events was still in doubt (1557, 4376).

In 1965 Quarterman (4671) injected D_3 into rats, rabbits, sheep, goats, and a pig, and observed chromatographic increases of a substance in the ileum, liver, kidneys, and adrenals that he suspected was a metabolite of D_3, although he could not prove it.

In 1966 Neville and DeLuca synthesized H^3-labeled D_3 of high specific activity (4376), and in the same year Lund and DeLuca (3613) and Fraser and Kodicek (1905) isolated at least three metabolites of D_3 that they described as esters.

In 1967 Morii, Neville and DeLuca (4072) showed that one of these metabolites, identified chromatographically as Peak IV, was as active as D_3 itself in rats, and faster at stimulating Ca transport.

In 1967 Loomis (3575, 3576) reiterated the proposal (see Park, 1923, above) that the vitamin D system was essentially endocrine, driven in man by UV irradiation, and controlled in the long term by the degree of skin pigmentation.

In 1968 Blunt, DeLuca and Schnoes unequivocally identified 25-OH-D_3 (0633); they synthesized it (0630), and DeLuca and his coworkers demonstrated that it was elaborated by a specific enzyme system in the liver (4376, 4598). The same team in 1969 isolated and characterized 25-OH-D_2 (4376, 5588).

Meanwhile, in 1968, Haussler, Myrtle and Norman demonstrated the existence of a D_3 metabolite that was more polar then 25-OH-D_3 and that was active in Ca transport (4376).

In 1969 Seelig reviewed the clinical literature and her own work (5216), concluding that in the general population of the United States the hazards of too much vitamin D activity often outweighed the hazards of rickets.

In 1969 Lawson, Wilson, and Kodicek used doubly-labeled D_3 with H^3 in the C-1α position to discover that the highly active, more polar metabolite of D_3 was in fact altered at that position (4376). In 1970 Fraser and Kodicek (1903) discovered that this metabolite was synthesized only in the kidney.

In 1971 Holick and coworkers in DeLuca's laboratory (2691, 2693) identified this metabolite as 1,25-$(OH)_2$-D_3. In 1972 Semmler and coworkers in the same laboratory achieved chemical synthesis of 1,25-$(OH)_2$-D_3, proving that it was in fact 1α,25-$(OH)_2$-D_3 (4376). In 1973 they prepared the potent synthetic analog, 1α-OH-D_3 (2688).

When Omdahl and DeLuca (4376) reviewed the state of the art in 1973, many metabolites and analogs of D_2, D_3, and tachysterol had been rapidly isolated, characterized, and synthesized. Studies with these compounds had led to identification of precise metabolic defects in several forms of D-resistant rickets and hypercalcemias secondary to kidney malfunctions. Prospects of specific treatments with low doses of these new compounds were being discussed in the literature.

Some of these compounds, notably 25-OH-D_3, 1α-OH-D_3, 1α,25-$(OH)_2$-D_3, and the isotachysterols had been found to act faster, more potently, or more selectively than the "vitamins" D. Theoretically, their margins of safety were expected to be smaller than those of the "vitamins" D. Toxicology studies had been started, but were not yet ready for publication (private communications). [Nevertheless, off-the-record opinions were received that at least one of these compounds, 25-OH-D_3, was being considered as a possible replacement for vitamin D as a food additive.]

Finally, the conclusions of the early investigators, Huldshinsky and Park, were confirmed by DeLuca and his coworkers. In 1972 DeLuca commented (1429) that D probably became a vitamin when man started to wear clothes; this of course was before air pollution (3577) became a problem. In 1973 Omdahl and DeLuca (4376) identified 1α,25-$(OH)_2$-D_3 as a hormone, and D_3 as a prohormone on the basis of specific evidence as to their chemistry, metabolism, distribution, and modes of action.

CHEMICAL INFORMATION

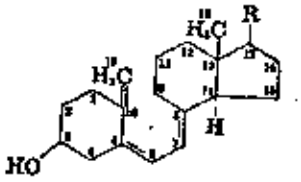
I. Nomenclature

A. Common Names

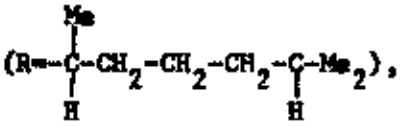
A system of nomenclature for the vitamin D compounds has been recommended by the International Union of Nutrition Sciences Committee on Nomenclature and the Committee on Nomenclature of the American Institute of Nutrition, as of January 1974 (0200), without change from their recommendations of January 1973 (0195):

1. "Vitamin D" means all steroids with the same sort of activity as vitamin D_3. Phrases such as "vitamin D activity" or "vitamin D deficiency" are preferred usage.

2. Three compounds are named specifically, all having the basic structure:

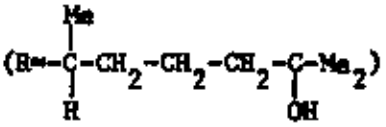


a. Vitamin D_3, with the following at R, should be called cholecalciferol.



b. Vitamin D_2, with the following at R, should be called ergocalciferol.

c. The compound with the following at R should be called 25-hydroxycholecalciferol.



3. Esters of cholecalciferol should be called cholecalciferyl esters, and esters of ergocalciferol should be called ergocalciferyl esters.

Together, the standard reference sources and classic review articles list many compounds with vitamin D activity and many names for some of these compounds, as follows:

Vitamin D_2: Ergocalciferol (6395), formerly calciferol (5877): both names given equal status elsewhere (1120, etc.), antirachitic factor (0071). Other names: oleovitamin D_2, viosterol, activated ergosterol (5511); irradiated ergosterol (1850).

Vitamin D_3: Cholecalciferol (6395, etc.), activated 7-dehydrocholesterol (5877, etc.). Other names: oleovitamin D_3 (5511). 7-dehydrocholesterol is also called provitamin D_3 (5878).

Vitamin D: Includes vitamins D_2 and D_3, and also the following (5511):

Salts of D_2 and D_3: *p*-nitrobenzoate; 3,5-dinitrobenzoate; phenylurethan; allophanate.

Vitamin D_4, or 22,23-dihydrovitamin D_2 and its 3,5-dinitrobenzoate salt.

Tachysterol and its 4-methyl-3,5-dinitrobenzoate salt.

Dihydrotachysterol, or dichystrolum (5511).

Lumisterol and its salts: acetate; 3,5-dinitrobenzoate; allophanate.

Phytosterol was not mentioned in the sources consulted.

In 1956 Kodicek, in a review (3208), noted the synthesis of a number of compounds related to D:

Triconjugated tachysterol analogs:

"All"-*trans*-10(5),6,8(14),22-tetraene,

"All"-*trans*-1(10),5,7,22-tetraene,

6,7-*cis*-Tachysterol.

Vitamin D analogs:

9,10-*seco*-10(19),5-*trans*,7-*cis*,22-*trans*-Ergostatetraene,

9,10-Dihydroxy,9,10-dihydro-precalciferol. (Evidence was cited that 6,7-*cis*-tachysterol and precalciferol were identical.)

Isotachysterol,

Isovitamin D_2, identical with pyrotachysterol,

μ-Tachysterol, or 4,6-*trans*,8(14),22-tetraene,

4,4-Dimethylcalciferol,

Four D_3-phosphate salts, and a D_3-lithium salt.

Other sterols with definite or possible D activity have been listed (5204):

Sitosterol and its 7-dehydro- form,

Stigmasterol and its 7-dehydro- form,

Brassicasterol,

Campesterol and its 7-dehydro- form,

Fucosterol,

Epiergosterol,

22,23-Oxidoergosterol,

22-Dihydroergosterol,

7-Dehydroclionasterol, or chondrillasterol (0071),

7-Dehydroepicholesterol,

$\Delta^{5,7,22}$-Cholestatriene-3-ol,

$\Delta^{5,7}$-Norcholestadiene-3-ol,

3-Hydroxy-$\Delta^{5,7}$-choladienic acid,

3,17-Dihydroxyandrostanediene,

And the esters listed in Tables 2 and 2a.

These lists were not claimed to be complete (5204). A more recent list (0071) includes vitamins D_2, D_3, and (with synonyms):

7-Dehydrostigmasterol (provitamin D, corbisterol)

Epi-7-dehydrocholesterol (provitamin D_1)

Ergosterol (provitamin D_2)

Lumisterol (provitamin D_2)

Tachysterol (provitamin D_2)

7-Dehydrocholesterol (provitamin D_3)

22,23-Dihydroergosterol (provitamin D_4)

7-Dehydrositosterol (provitamin D_5)

A growing list of newly identified or synthesized compounds with vitamin D activity includes:

25-Hydroxyvitamin D_2 (5587)

25-Hydroxyvitamin D_3 (0630)

1,25-Dihydroxyvitamin D_2 (4376)

1α,25-Dihydroxyvitamin D_3 (2688, 4302)

24,25-Dihydroxyvitamin D_3 (4376)

25,26-Dihydroxyvitamin D_3 (5586)

1α-Hydroxyvitamin D_3 (2688)

5,6-*trans*-Vitamin D_3 (2688)

Isovitamin D_3 (2689)

Isotachysterol$_3$ (2689)

25-Hydroxyisotachysterol$_3$ (2689)

Dihydrotachysterol$_3$ (4376)

25-Hydroxydihydrotachysterol$_3$ (4376)

This list is believed to be incomplete.

Table 2

Ergosterol Esters (5204)

Ester	Melting point, °C	Rotation (in $CHCl_3$)
Ergosteryl acetate	179 turbid	$[\alpha]_D^{25} = -90°$
	181 clear	
Ergosteryl allophanate	250	
Ergosteryl β-anthraquinone carbonate	195-200	
Ergosteryl benzoate	169-171.5	$[\alpha]_{5461}^{20} = -88.3°$
Monoergosteryl esters of n-butane-1,2,3,4-tetracarboxylic acid:		
the more soluble isomer	168 decomp.	
the less soluble isomer	230 decomp.	
Ergosteryl butyrate	100-129.5	$[\alpha]_n = -73°$
Ergosteryl 2-chloro-3,5-dinitrobenzoate	203-204	$[\alpha]_D^{25} = -38°$
Ergosteryl cinnamate	175 turbid	$[\alpha]_D^{19} = -50.8°$
	190 clear	
Ergosteryl 3,5-dinitrobenzoate	202	
Ergosteryl 3,5-dinitrobenzoate	198-199	$[\alpha]_n = -40.8°$
Ergosteryl 3,5-dinitro-4-methyl-benzoate	213-214	$[\alpha]_D^{20} = -49°$
Ergosteryl diphenylacetate	186	$[\alpha]_D^{17} = -60°$
Ergosteryl ethyl carbonate	150-153.5	$[\alpha]_{5461}^{20} = -111.1°$
Ergosteryl formate	161.5	$[\alpha]_n = -97.9°$
Ergosteryl isobutyrate	148 viscous, turbid	$[\alpha]_D^{25} = -84°$
	159 thin, turbid	
	162 clear	
Ergosteryl isovalerate	138 viscous, turbid	$[\alpha]_D^{25} = -82°$
	157 thin, turbid	
	160 clear	
Ergosteryl β-naphthoate	175	

Table 2 (cont.)

Ester, reference	Melting point, °C	Rotation (in $CHCl_3$)
Ergosteryl α-naphthylurethane	186	$[\alpha]_D^{16} = -55°$
Ergosteryl 3-nitrobenzoate	151	$[\alpha]_D = -71°$
Ergosteryl 4-nitrobenzoate	182	$[\alpha]_D = -49.5°$
Ergosteryl 3-nitro-4-methylbenzoate	191-193	$[\alpha]_D = -47.2°$
Diergosteryl oxalate	255	$[\alpha]_D^{20} = -76.4°$
Ergosteryl palmitate	107-108	$[\alpha]_D^{15} = -50.9°$
Ergosteryl phenylurethane	185	$[\alpha]_D^{16} = -63.1°$
Diergosteryl [β-chloroethyl] phosphate	165-167	
Diergosteryl phosphate	180-182	
Monoergosteryl phosphite	146	
Monoergosteryl phthalate	169	$[\alpha]_D = -51°$
Diergosteryl propionate	147.5	$[\alpha]_D = -77°$
Monoergosteryl succinate	162	

Table 2a

Vitamin D Esters (5204)

Ester	Melting point, °C	Specific rotation
Calciferyl (Vitamin D_2) acetate	86	$[\alpha]_{5790}$ = +38° acetone
Calciferyl allophanate	194-195	$[\alpha]_D^{20}$ = +50.4° chloroform
Calciferyl anisate	99.5-101	$[\alpha]_D^{21}$ = +120° chloroform
Calciferyl benzoate	92	$[\alpha]_{5790}$ = +100° acetone
Monocalciferyl esters of *n*-butane-1,2,3,4-tetracarboxylic acid (unseperated isomers)	90-100 decomp.	
Calciferyl chaulmoograte	53	$[\alpha]_{5790}$ = +52° chloroform
Calciferyl 2-chloro-3,5-dinitrobenzoate	132	$[\alpha]_D^{22}$ = +60° acetone
Calciferyl 3,5-dinitrobenzoate	147-149	$[\alpha]_{5461}^{20}$ = +69° benzene
Calciferyl 3,5-dinitrobenzoate	146-147	$[\alpha]_D^{20}$ = +91.5° chloroform
Calciferyl 3,5-dinitrobenzoate		$[\alpha]_D^{20}$ = +86.5° acetone
Calciferyl 3,5-dinitro-4-methylbenzoate	116-117	$[\alpha]_D^{20}$ = +95.8° chloroform
Calciferyl β-naphthoate	132	$[\alpha]_D^{20}$ = +150° chloroform
Calciferyl 4-nitrobenzoate	94.5-95	$[\alpha]_D^{20}$ = +105.2° chloroform
Calciferyl 3-nitro-4-methylbenzoate	119-120	$[\alpha]_D^{20}$ = +106.8° chloroform
Calciferyl oleate	Liquid	$[\alpha]_{5790}$ = +13.7° chloroform
Calciferyl phenylurethane	122	$[\alpha]_D^{19}$ = +49.2° chloroform
Calciferyl propionate	77	$[\alpha]_{5790}$ = +37.6° acetone
Vitamin D_3 anisate	114	$[\alpha]_D^{21}$ = +127° chloroform

Table 2a (cont.)

Ester	Melting point, °C	Specific rotation
Vitamin D_3 3,5-dinitrobenzoate (dimorphous):		
from benzene-methanol	132	$[\alpha]_D^{20}$ = +97.9° chloroform
from ether	141	
leaflets	142	$[\alpha]_D^{20}$ = +97° chloroform
needles	150	
Vitamin D_3 3,5-dinitro-4-methylbenzoate	128-129	$[\alpha]_D^{20}$ = +106.6° chloroform
Vitamin D_3 4-nitrobenzoate	125-126	$[\alpha]_D^{20}$ = +114.6° chloroform
Vitamin D_4 3,5-dinitrobenzoate	127-128	$[\alpha]_D^{22}$ = +93.2° acetone
Vitamin D_m 3,5-dinitrobenzoate	128-128.5	$[\alpha]_D$ = +92° chloroform

B. Chemical Names

Systematic names were found for the following compounds mentioned in the preceding section, with occasional differences between sources, which are cited accordingly:

Common name	Systematic name(s)
Vitamin D_2	9,10-Secoergosta-5,7,10(19)-tetraen-3-ol (0071)
Vitamin D_3	22,23-Dihydro-24-demethyl-calciferol (0071)
Stigmasterol	(24*S*)-24-Ethylcholesta-5,22-dien-3β-ol (0071)
	Stigmasta-5,22-dien-3β-ol (6393)
7-Dehydrostigmasterol	(24*S*)-24-Ethylcholesta-5,7,22-trien-3β-ol (0071)
	Stigmasta-5,7,22-trien-3β-ol (6393)
Dihydrotachysterol	9,10-Secoergosta-5,7,22-trien-3β-ol (5511)
Lumisterol	9β,10α-Ergosta-5,7,22-trien-3β-ol (6393)
Sitosterol	Stigmast-5-en-3β-ol (6393)
7-Dehydrositosterol	Stigmast-5,7-dien-3β-ol (6393)
7 Dehydrocholesterol	Cholesta-6,7-dien-3β-ol (0071, 6393)
Ergosterol	Ergosta-5,7,22-trien-3β-ol (0071, 6393)
22,23-Dihydroergosterol	(24*S*)-24-Methylcholesta-5,7-dien-3β-ol (0071)
Brassicasterol	(24*R*)-24-Methylcholesta-5,22-dien-3β-ol (0071)
	Ergosta-5,22-dien-3β-ol (6393)
Campesterol	(24*R*)-24-Methylcholest-5-en-3β-ol (0071)
	Ergost-5-en-3β-ol 24-epimer (6393)
Fucosterol	(*E*)-24-Ethylidenecholest-5-en-3β-ol (0071)
	Stigmasta-5,24(28)-dien-3β-ol (6393)
7-Dehydroclionasterol	(24*S*)-24-Ethylcholesta-5,22-dien-3β-ol (0071)
$\Delta^{5,7,22}$-Cholestatriene-3-ol	Cholesta-5,7,22-trien-3β-ol (0071)

C. Trade names (5511)

Vitamin D_2:

Condol	Diactol	Infron
D-Tracetten	Divit Urto	Metadee
Davitin	Doral	Mina D_2
Decaps	Drisdol	Mulsiferol
Dee-Ron	Ergorone	Mykostin
Deltalin	Ertron	Ostelin
Deratol	Fortodyl	Panvitan (6393)
Detalup	Hi-Deratol	Radiostol

Radsterin	Deparal	Dihydrotachysterol:
Shock-Ferol	Ebivit	A.T.10
Sterpyl	Neo Dohyfral D_3	Antitanil
Tongevit (6393)	Ricketon	Anti-tetany Substance 10
Vio-D	Trivitan	Calcamine
Vitamin D_3:	Vi-De-3-hydrosol	Hytakerol
D_3-Vicotrat	Vigantol	Parterol
Delsterol	Viprosan	

D. Chemical Abstracts Services Unique Registry Numbers:

Vitamin D_2 50140

Vitamin D_3 67970

Vitamin D 1406162

II. Empirical Formulas (6511)

Vitamin D_2: $C_{28}H_{44}O$

Salts: p-nitrobenzoate: $C_{35}H_{47}NO_4$

3,5-dinitrobenzoate: $C_{35}H_{46}N_2O_6$

phenylurethan: $C_{35}H_{49}NO_2$

allophanate: $C_{30}H_{46}N_2O_3$

Vitamin D_3: $C_{27}H_{44}O$

Salts: p-nitrobenzoate: $C_{34}H_{47}NO_4$

3,5-dinitrobenzoate: $C_{34}H_{46}N_2O_6$

allophanate: $C_{29}H_{46}N_2O_3$

Vitamin D: Vitamin D_4: $C_{28}H_{44}O$

3,5-dinitrobenzoate salt: $C_{35}H_{48}N_2O_6$

Tachysterol: $C_{28}H_{44}O$

4-methyl-3,5-dinitrobenzoate salt: $C_{36}H_{48}N_2O_6$

Dihydrotachysterol: $C_{28}H_{46}O$

Lumisterol: $C_{28}H_{44}O$

Further empirical formulas (0071):

Stigmasterol	$C_{29}H_{48}O$
7-Dehydrostigmasterol	$C_{29}H_{46}O$
7-Dehydrositosterol	$C_{29}H_{48}O$
Epi-7-dehydrocholesterol	$C_{27}H_{44}O$

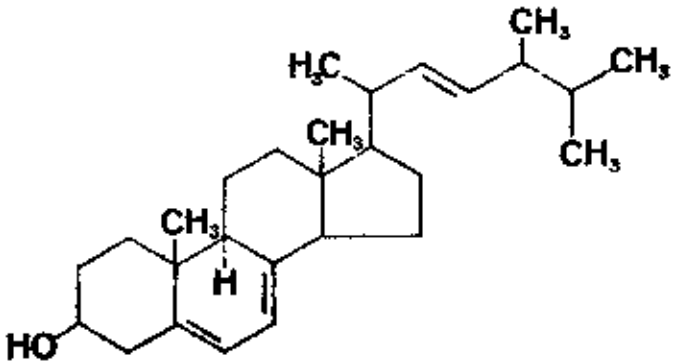
7-Dehydrocholesterol	$C_{27}H_{44}O$
Ergosterol	$C_{28}H_{44}O$
22,23-Dihydroergosterol	$C_{28}H_{46}O$
Brassicasterol	$C_{28}H_{46}O$
Campesterol	$C_{28}H_{48}O$
Fucosterol	$C_{29}H_{48}O$
7-Dehydroclionasterol	$C_{29}H_{48}O$
$\Delta^{5,7,22}$-Cholestatriene-3-ol	$C_{27}H_{42}O$
25-Hydroxyvitamin D_3	$C_{27}H_{44}O_2$ (4302)
1,25-Dihydroxyvitamin D_3	$C_{27}H_{44}O_3$ (4302)

The above list is partial. The empirical formula of a D-active compound does not identify it chemically, nor does it indicate the presence or absence of D activity. Better identification is provided by the structural formulas (below).

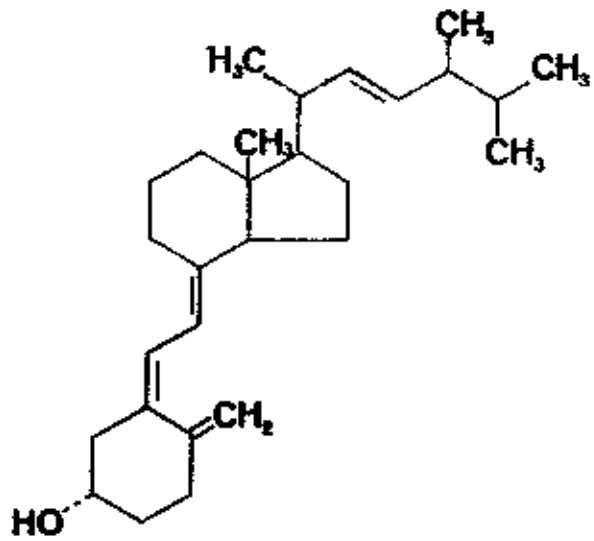
III. Structural Formulas

Structures are given in the following pages (no Fig. numbers) for a selection of the compounds named in the preceding sections. They are drawn in the manner adopted by two teams currently occupied in characterizing D-active compounds (led respectively by Prof. Lodicel and H.F. DeLuca). This manner of drawing permits, for example, rotations of the A ring of the steroid nucleus to be illustrated.

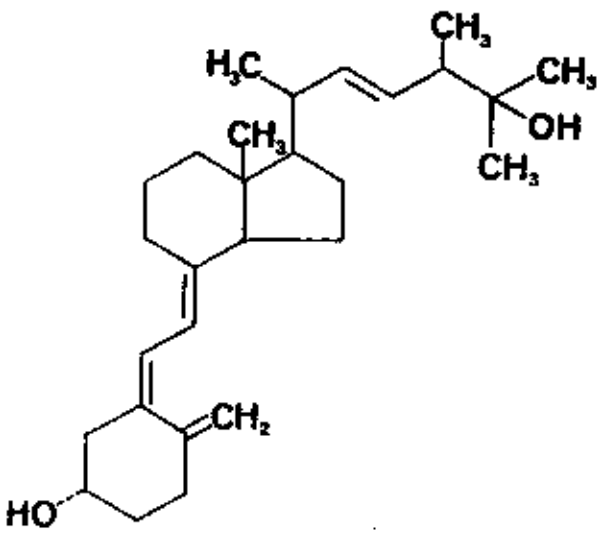
Ergosterol



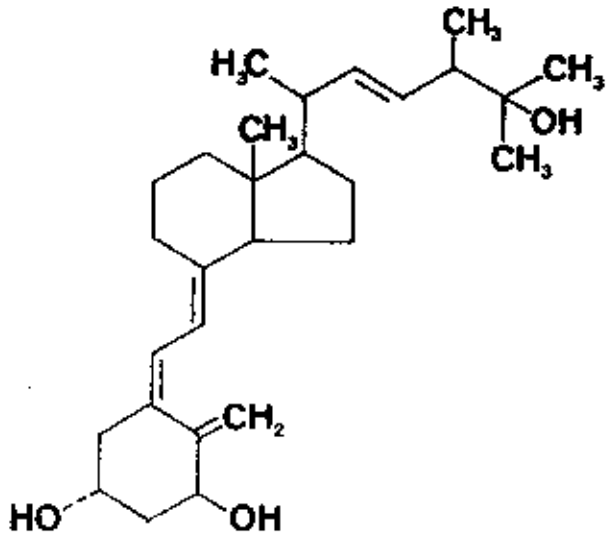
Vitamin D_2



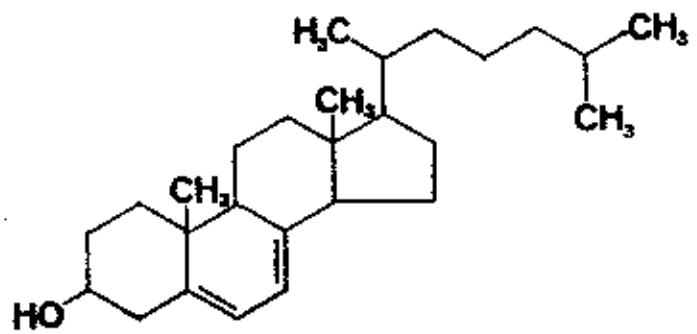
25-Hydroxyvitamin D_2



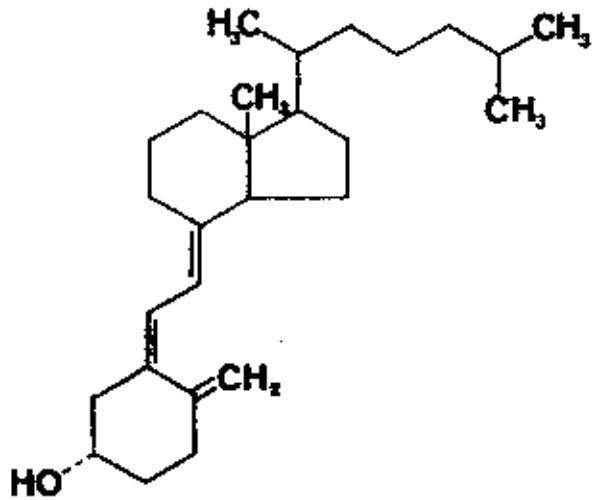
1α,25-Dihydroxyvitamin D_2



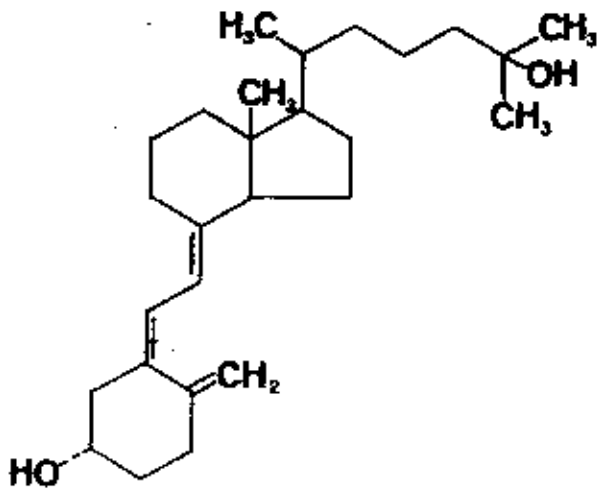
7-Dehydrocholesterol



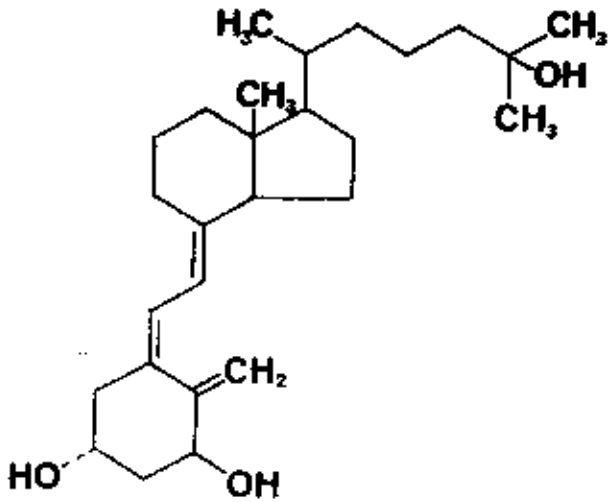
Vitamin D_3



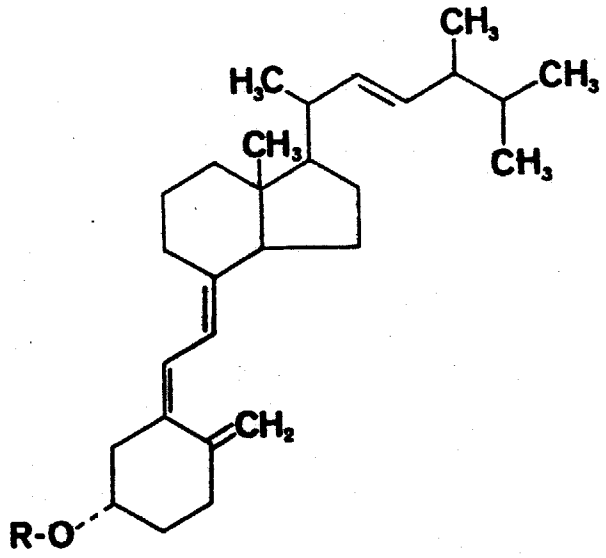
25-Hydroxyvitamin D_3



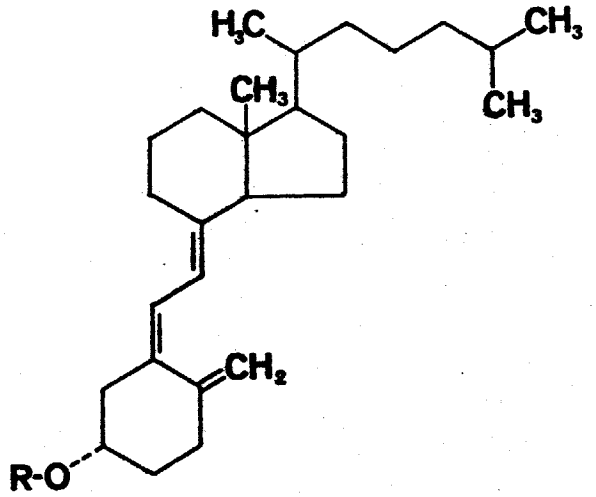
1α,25-Dihydroxyvitamin D_3



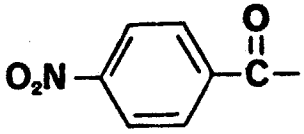
Esters of D_2 and D_3



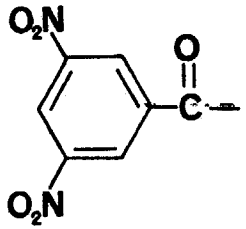
Vitamin D_2

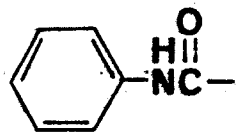


Vitamin D_3

p-Nitrobenzoate, R= 

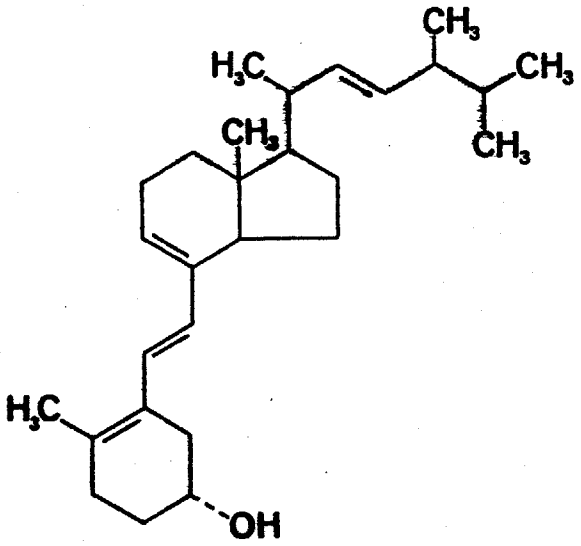
Allophanate, R=

3,5-Dinitrobenzoate, R= 

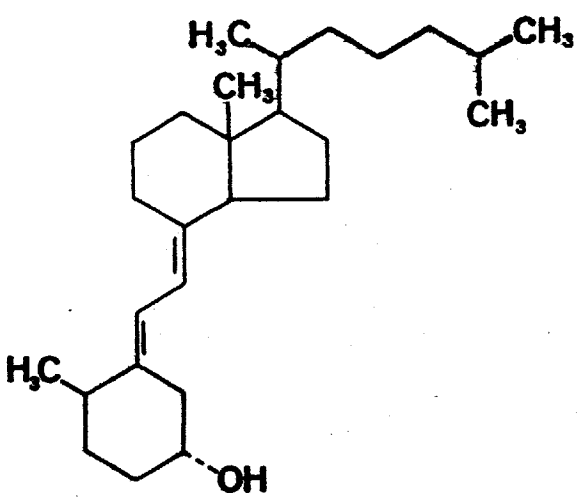
Phenylurethan, R= 

Vitamin D_2 Only

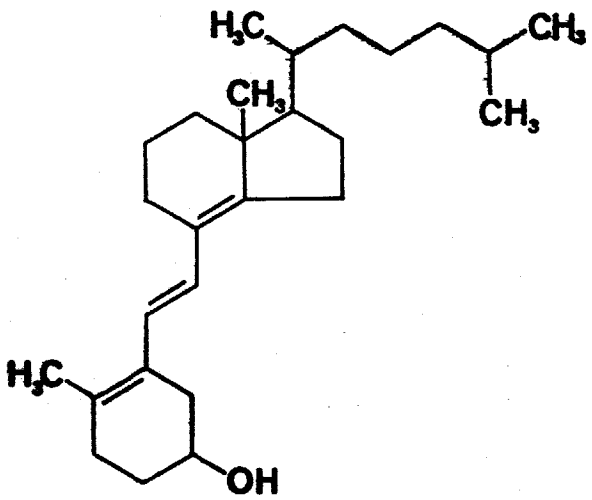
Tachysterol



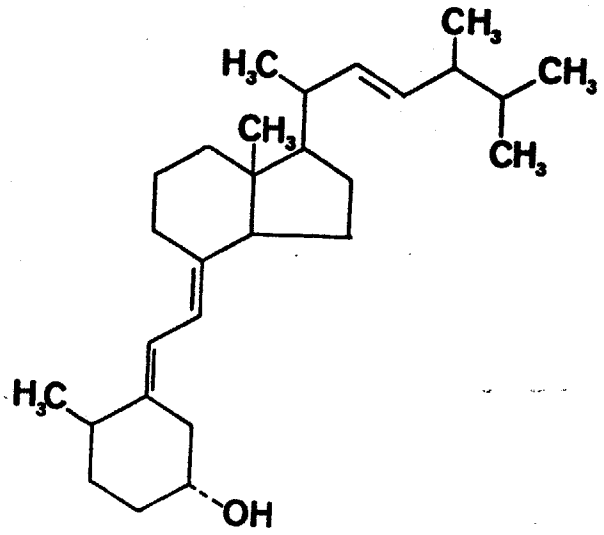
Dihydrotachysterol$_3$



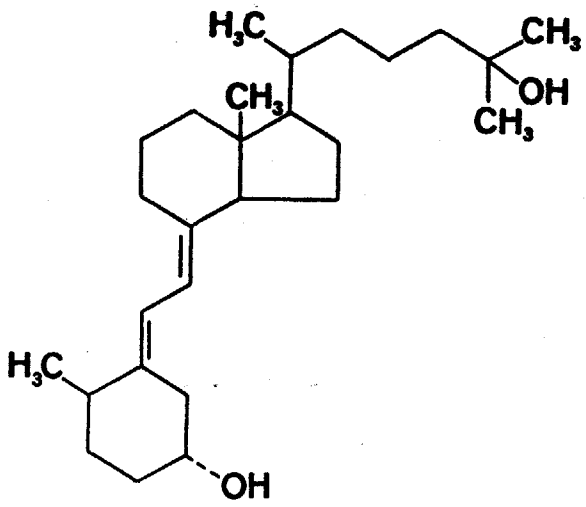
Isotachysterol$_3$



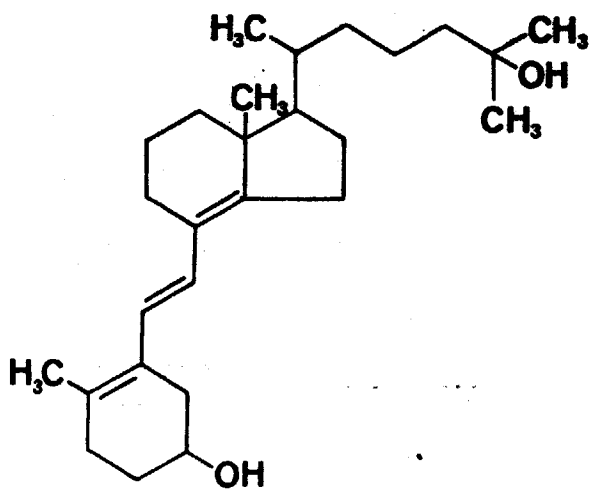
Dihydrotachysterol



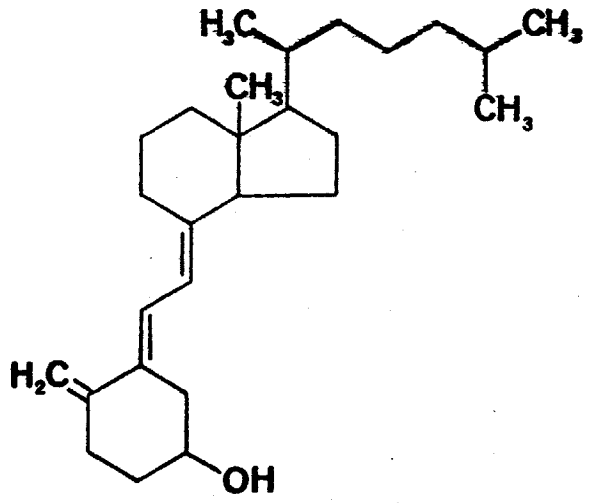
25-Hydroxydihydrotachysterol$_3$



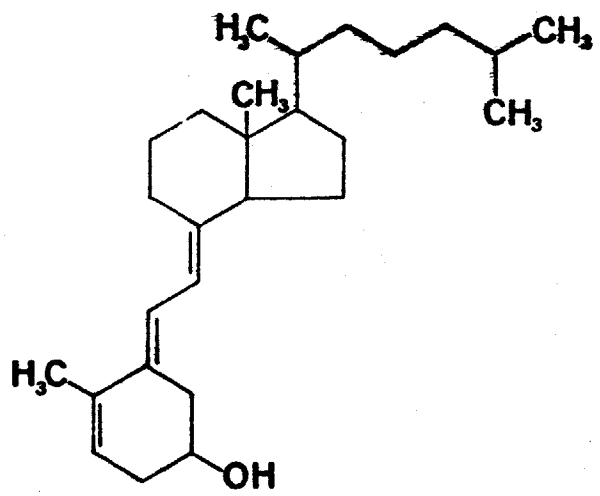
25-Hydroxyisotachysterol$_3$



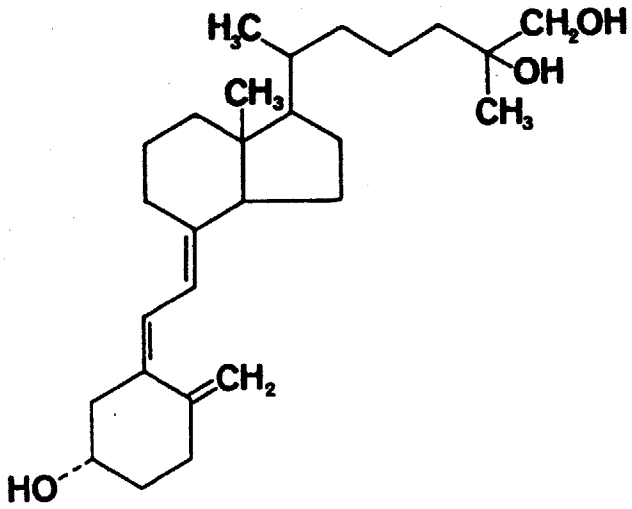
5,6-*trans*-Vitamin D_3



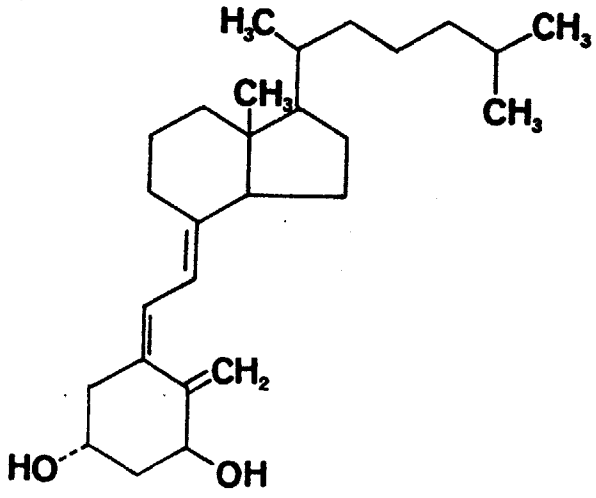
Isovitamin D_3



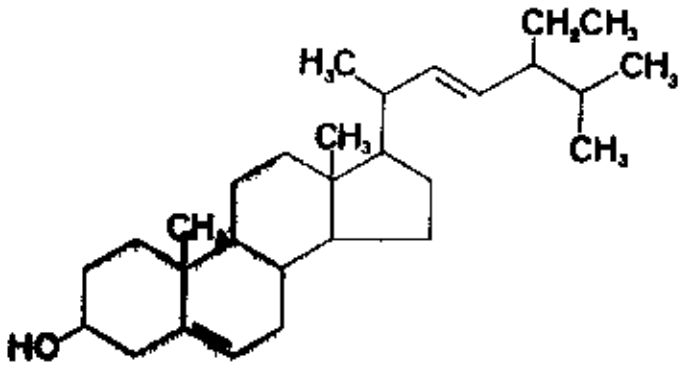
25,26-Dihydroxyvitamin D_3



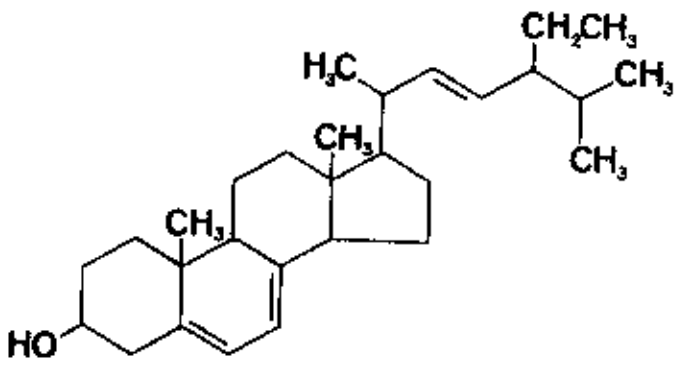
1α-Hydroxyvitamin D_3



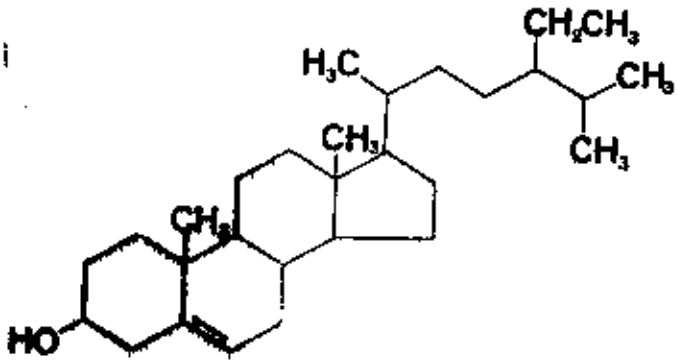
Stigmasterol



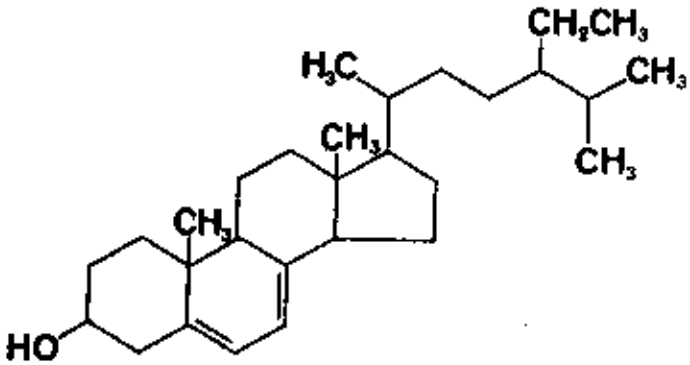
7-Dehydrostigmasterol



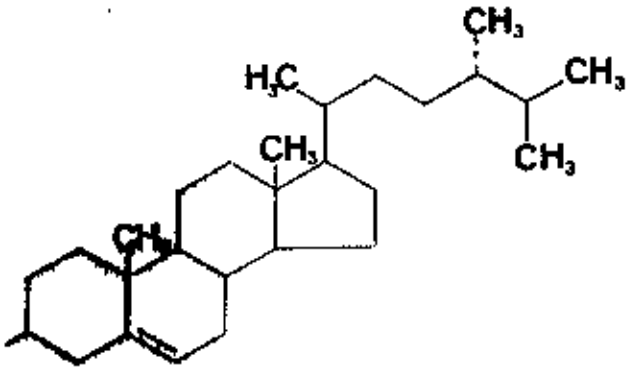
Sitosterol



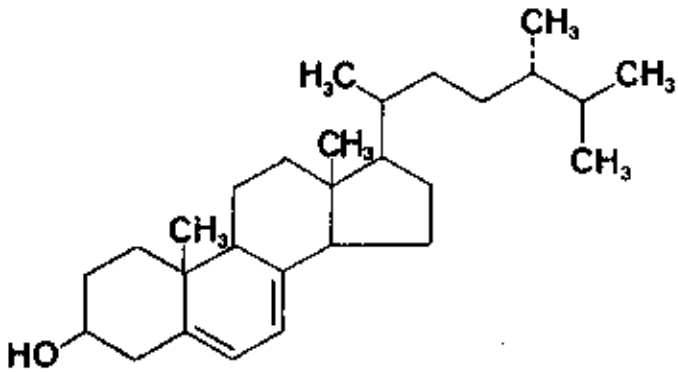
7-Dehydrositosterol



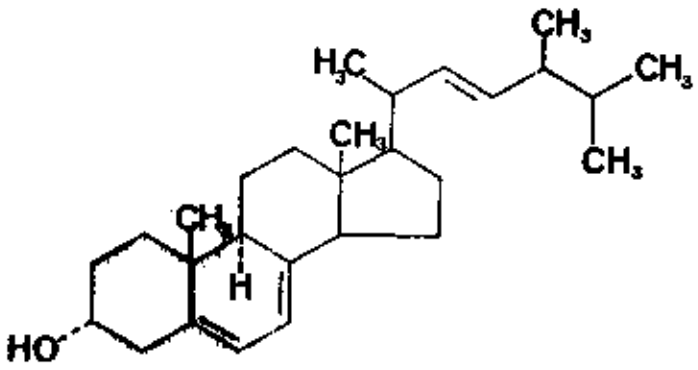
Campesterol



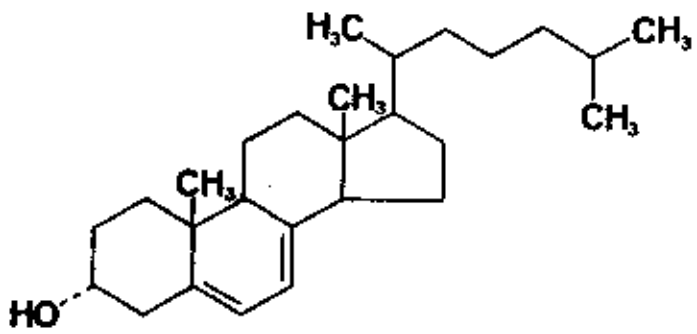
7-Dehydrocampesterol



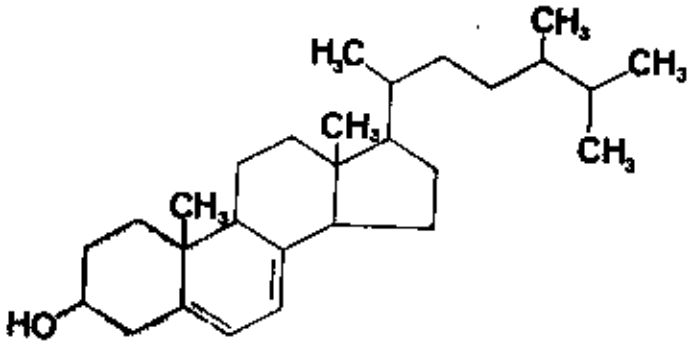
7-Dehydroergosterol



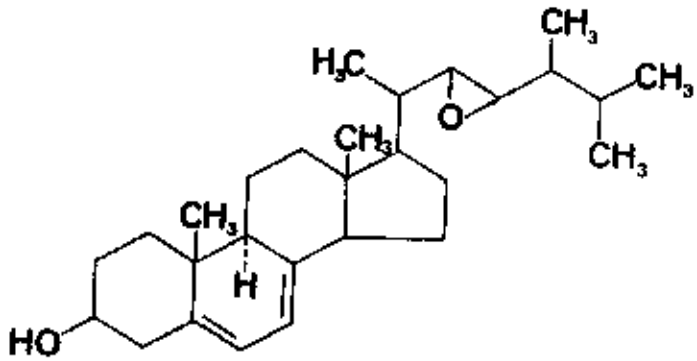
7-Dehydroepicholesterol



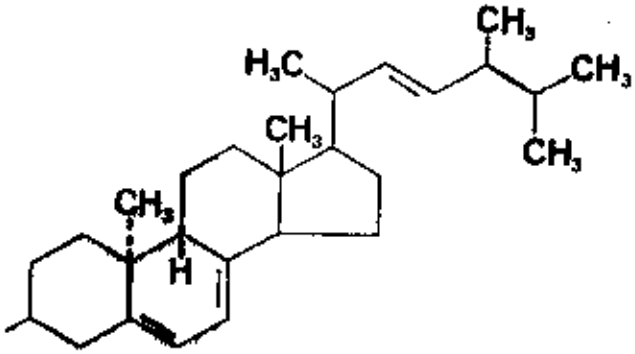
22,23-Dihydroergosterol



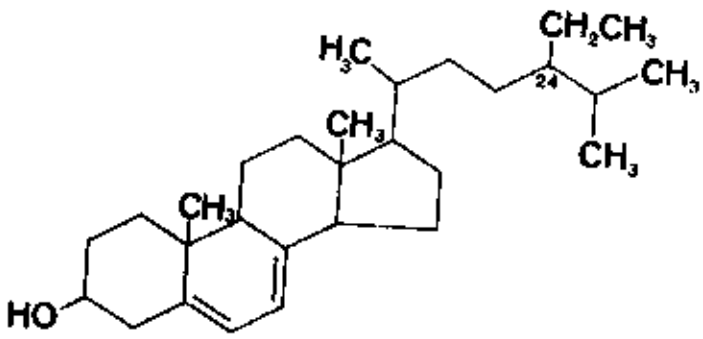
22,23-Oxidoergosterol



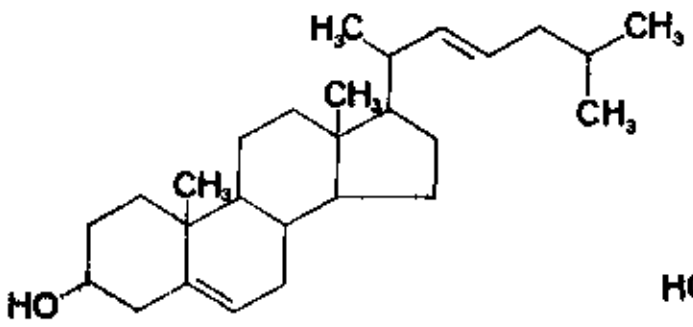
Lumisterol



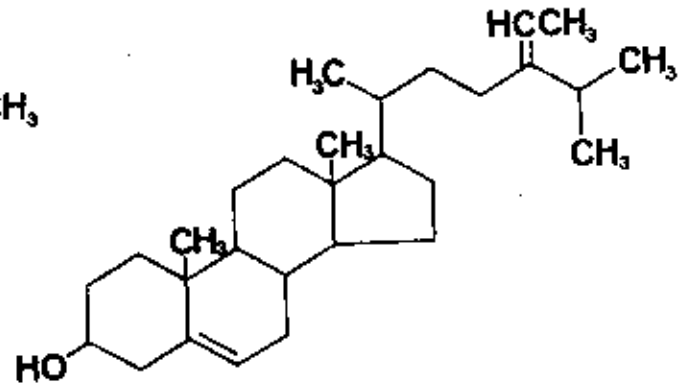
7-Dehydroclionasterol



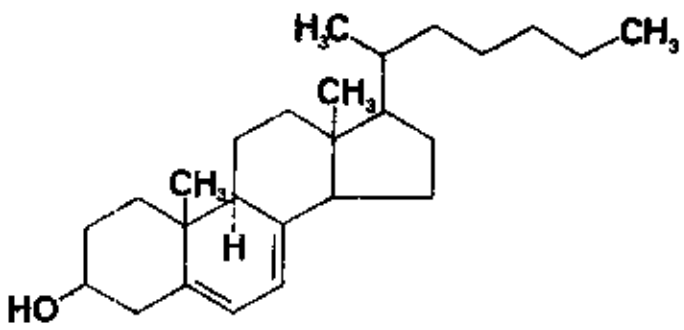
Brassicasterol



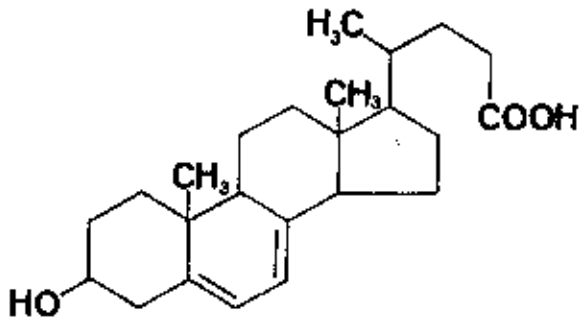
Fucosterol



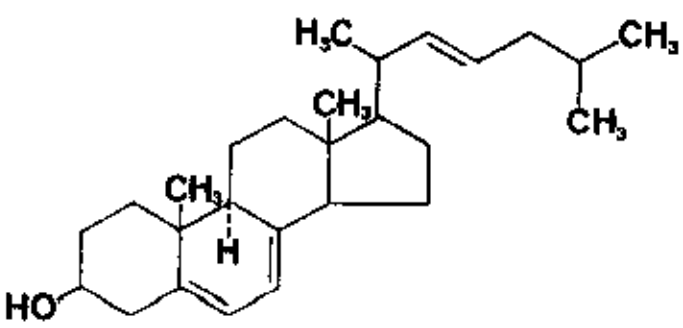
$\Delta^{5,7}$-Norcholestadiene-3-ol



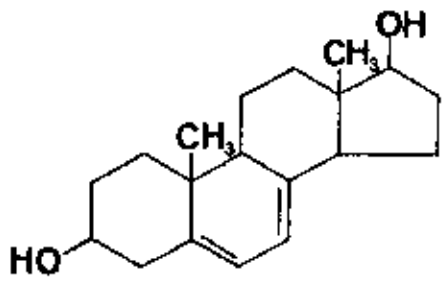
3-Hydroxy-$\Delta^{5,7}$-choladienic acid



$\Delta^{5,7,22}$-Cholestatriene-3-ol



3,17-Dihydroxyandrostanediene



XV. Molecular Weights

Vitamin D_2 396.63

p-nitrobenzoate 445.78

3,5-dinitrobenzoate 590.77

phenylurethan 515.79

allophanate 482.71

Vitamin D_3 384.62

p-nitrobenzoate 533.71

3,5-dinitrobenzoate 578.75

allophanate 470.70

Vitamin (provitamin) D_4 398.65

3,5-dinitrobenzoate 593.78

Stigmasterol 412.70

7-Dehydrostigmasterol 410.69

Tachysterol 396.66

4-methyl-3,5-dinitrobenzoate 592.78

Dihydrotachysterol 398.68

Lumisterol 396.66

Sitosterol 414.69

7-Dehydrositosterol 412.70

Epi-7 dehydrocholesterol 384.65

7-Dehydrocholesterol 384.65

Ergosterol 396.66

Brassicasterol 398.98

Campesterol 400.99

7-Dehydrocampesterol 398.68

Fucosterol 412.70

Epiergosterol 396.66

22,23-Oxidoergosterol 412.66

22-Dihydroergosterol 398.65

7-Dehydroclionasterol 412.70

7-Dehydroepicholesterol 384.62

$\Delta^{5,7,22}$-Cholestatriene-3-ol 382.64

$\Delta^{5,7}$-Norcholestadiene-3-ol 370.62

3-Hydroxy-$\Delta^{5,7}$-cholandienic acid 371.54

3,17-Dihydroxyandrostanediene 287.42

25-Hydroxyvitamin D_2 417.33 (5587)

25-Hydroxyvitamin D_3 400 (4302)

1,25-Dihydroxyvitamin D_2 428.63

1,25-Dihydroxyvitamin D_3 416 (4302)

1α,25-Dihydroxyvitamin D_2 428.63

1α 25-Dihydroxyvitamin D_3 416.62

25,26-Dihydroxyvitamin D_3 416.62

1α-Hydroxyvitamin D_3 400.62

5,6-*trans*-Vitamin D_3 384.62

Isovitamin D_3 384.65

Isotachysterol$_3$ 384.65

25-Hydroxyisotachysterol$_3$ 400.65

V. Specifications

Vitamin D is specified in two ways:

(1) Bioassay, the reference standard being the biological activity shown in rats by 0.025 μg of D_3, defined as one IU.

(2) Chemical analysis defining the reference standard.

Many sterols naturally occurring in foodstuffs are activated by UV of appropriate wavelengths, and the bioassay does not separate their activities from that of added D_2 or D_3. Activation results in metabolism and the end-products are one or more hormones (see Section on Biochemical Aspects) that the bioassay does not identify. Bioassay of foodstuffs to which D_2 or D_3, known chemical compounds, have been added specifies only total activity, known as Vitamin D activity (see Nomenclature).

Numerous compounds capable of being activated have been listed elsewhere in this Section. Complete information on this capability was not found, for example, on all steroids or sterols occurring in foodstuffs or added to them for other purposes.

Obviously, tests for total activity do not specify chemical purity, in the case of the vitamins D. However, when D_2 or D_3 can be isolated, for example from a chemically defined preparation, tests of identity are specified (6395).

Very recently the pure hormonal forms of several vitamins D have been isolated, synthesized, and found capable of being synthesized in large quantities, as have synthetic compounds with direct D activity. However, no specifications for any of these substances as food additives were found.

Specifications of D activity have altered little since they were reviewed in 1938 by Nelson (4212). According to him:

Before 1934 three different units of D activity were used in the U.S.A.: (1) the Steenbock Unit, (2) the A.D.M.A. Unit, and (3) the Poulsson or Oslo Unit. In 1931 the Health Organisation of the League of Nations adopted a British unit based on a chemically defined quantity of D_3 as an International Standard, expressed as an International Unit of activity (IU). The IU was defined as:

> "The vitamin D activity of 1 milligram of the International standard solution of irradiated ergosterol, which has been found equal to that of 0.025 microgram of crystalline vitamin D."

Conditions of preparation in a carrier, specified as olive oil, and of testing were described.

The 1934 Interim Revision of the USP adopted a specimen of cod liver oil as the "USP (Pharmacopoeia) Reference Cod Liver Oil" and defined the USP Unit of vitamin D as:

> "Equal in antirachitic potency for the rat, to one International Unit of Vitamin D as defined and adopted by the Conference of Vitamin Standards of the Permanent Commission on Biological Standardization of the League of Nations in June of 1931."

This issue of the USP also specified the conditions for raising the test animals, their numbers, age, weight limits, feeding methods, limits of the period for producing rickets, duration of assays, conduct of the line test, its criteria, the data required, and the methods of calculation (4212).

By 1938 the USP contained potency standards for three preparations:

Cod Liver Oil: "at least" 85 USP Units/g, or 312 USP Units/tsp.

Emulsion of Cod Liver Oil: 50% of the potency of Cod Liver Oil, v/v

Emulsion of Cod Liver Oil With Malt: 30% of the potency of Cod Liver Oil, v/v.

These three and no other preparations were named in the Federal Food and Drugs Act which required only that drugs should meet the potencies claimed for them (4212).

Since 1934, therefore, the formal specification of the reference standard has been revised from 0.025 µg of "crystalline vitamin D" to 0.025 µg of D_3, or cholecalciferol.

Some manufacturer specifications for D_2 and D_3 as supplied for fortification purposes and for formulation in vitamin supplements are included among the documents attached to this monograph (4511, 4863).

The USP bioassay potency of synthetic 25-OH-D_3 is reported as 55-60 IU/μg (4630), the authors noting that it was faster-acting than the standard compound.

VI. Description

Sections A, B, and C, Physical Properties, Stability, and Other Characteristics, are collected under each compound in turn. Data are taken from various authorities (0071, 0980, 1120, 5511).

Vitamin D_2:

Prisms from acetone (0071); white odorless crystals (1120).

mp 115-118°. Sublimes in high vacuum 0.0006 mm without decomposition.

Solubility:

acetone 7°	6.95 g/100 ml
organic solvents	soluble
vegetable oils	slightly soluble
water	insoluble

Not precipitated by digitonin.

Stability: affected by air and light.

crystals in evacuated amber ampuls 4° - 9 months

nitrobenzoic esters in these ampuls at room temperatures - 5 years

in dry propylene glycol, therapeutic concs., in amber screwcap bottles, sealed in air, 38° - 53 months

in corn oil, 38° - 30 months

Potency of crystals: 40 USP units/μg

Preparation: from ergosterol, in suitable solvent, by UV irradiation (275-300 mm) or longer-wave electron bombardment.

Absorption: max 264.5 nm in *n*-hexane.

specific $E^{1\%}_{1\,cm} = 458.9 \pm 7.5$

Optical rotations: $[\alpha]_D^{20}$ (see Table 5)

in alcohol	+102.5°
chloroform	+52°
petrolether	+33.3°
ether	+91.2°

$[\alpha]_D^{25}$	0.03g/1 ml acetone	+82.6°
$[\alpha]_{546}^{21}$	in acetone	+98.6°
$[\alpha]_{546}^{20}$	in alcohol	+119 to +122°

Salts of D_2:

p-nitrobenzoate: Pale yellow crystals from alcohol
mp 90-93°
Optical rotation: $[\alpha]_D^{20}$ 0.05 g/1 ml chloroform +104°

3,5-dinitrobenzoate: Yellow prisms from alcohol-chloroform
mp 149-149°
Optical rotations: $[\alpha]_D^{25}$ 0.05 g/1 ml acetone +80°
$[\alpha]_{546}^{20}$ in acetone +102°

phenylurethan: mp 122°
Optical rotation: $[\alpha]_D^{19}$ in chloroform +49.2°

allophanate: mp 194-195°
Optical rotation: $[\alpha]_D^{20}$ in chloroform +59.4°

Vitamin D_3:

Fine needles from acetone
mp 84-85°
Solubility: organic solvents soluble
vegetable oils slightly soluble
water practically insoluble
Not precipitated by digitonin
Stability: oxidized and inactivated by moist air in a few days
crystals in evacuated amber ampuls 4° - 1 year
nitrobenzoic ester in these ampuls
at room temperatures - 5 years
in dry propylene glycol, therapeutic
concs., in amber screwcap bottles,
sealed in air, 38° - 36 months
in corn oil, 38° - 30 months
At least as stable as D_2 (0071): considered more stable than D_2 (5511), or "generally somewhat more stable" (0988).
Potency: One USP unit or one IU is the activity of 0.025 µg of vitamin D_3 contained in the USP Reference Standard for vitamin D.

Preparation: (1) separated from fish liver oils by chromatography, molecular distillation, esterification and fractionation of the esters, etc.
(2) by UV irradiation of 7-dehydrocholesterol.

Absorption: max. in alcohol or n-hexane 264.5 nm
specific $E^{1\%}_{1\,cm}$ = 450–490

Optical rotations: $[\alpha]_D^{20}$ 1.6% w/v in acetone +84.8°
1.6% in chloroform +51.9°

Mass spectrum, see fig. 2 (4302)

Salts of D_3:

p-nitrobenzoate: Light yellow prisms from acetone
mp 127°
Absorption: max. 261 nm
Optical rotations: $[\alpha]_D^{20}$ 1.6% in acetone +116.4°
1.6% in chloroform +114.6°

3,5-dinitrobenzoate: Yellow needles from acetone
mp 129°
Absorption max. 265 nm
Optical rotation: $[\alpha]_D^{20}$ 1.6% in chloroform +100.0°

allophanate: Crystals from acetone
mp 173–174°

Vitamin D_4:

mp 96–98° (originally thought to be 107–108°) (5511).

Solubility: organic solvents soluble except in petrolether
vegetable oils slightly soluble
water practically insoluble

Not precipitated by digitonin

Preparation: from 22,23-dihydroergosterol by irradiation with Mg-arc light.

Absorption max. 265 nm

Optical rotation: $[\alpha]_D^{18}$ 9.4 mg/2 ml acetone +89.3°

Salts of D_4:

3,5-dinitrobenzoate: mp 127–128°

Optical rotation: $[\alpha]_D^{18}$ 9.1 mg/2 ml acetone +94.5°

Tachysterol:

mp and physical states not given

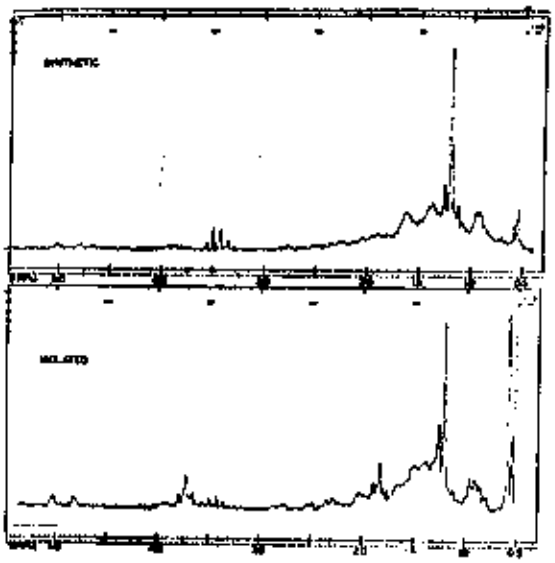


Fig. 1a. Nuclear magnetic resonance spectra of synthetic and isolated 25-hydroxycholecalciferol. Note that Δ = 0.58 ppm peaks are due to tetramethylsilane internal standard. (0630)

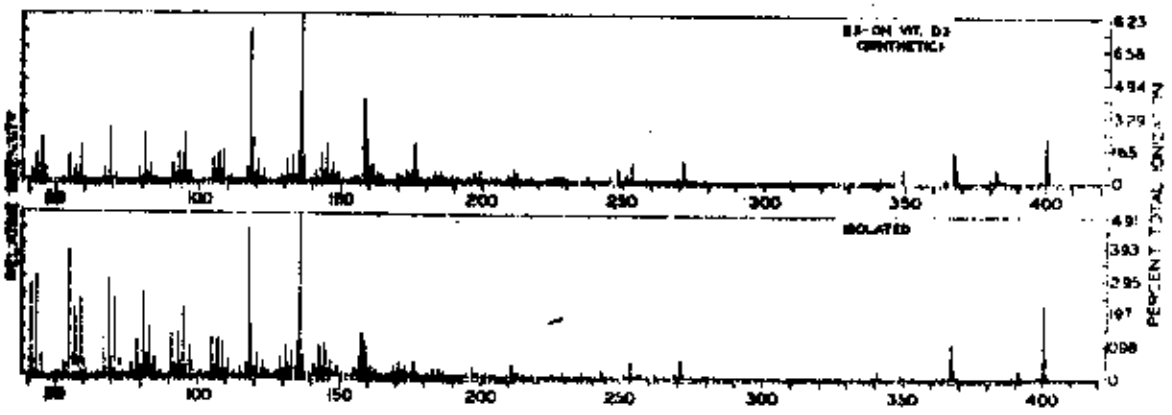


Fig. 1b. Mass spectrum of synthetic and isolated 25-hydroxycholecalciferol. (0630)

Solubility: organic solvents soluble except in methanol
fats very soluble
water insoluble

Very easily oxidized by air

Not precipitated by digitonin

Stability: not given

Preparation: (1) from ergosterol or lumisterol by UV irradiation
(2) from calciferol by adsorption on acid clay
(3) from "precalciferol"

Absorption max. 280 nm

Optical rotations: $[\alpha]_D^{18}$ 24.6 mg/2 ml petrolether - 70°
(oil solutions) $[\alpha]_{546}^{18}$ in petrolether - 86.3°

Salts of tachysterol:

4-methyl-3,5-dinitrobenzoate mp 155°; no other data

Dihydrotachysterol:

mp 125-127°

Solubility: organic solvents easily soluble

Preparation: by reduction of tachysterol

Absorption max. 242 nm

Optical rotation: $[\alpha]_D^{22}$ in chloroform +97.5°

Lumisterol:

Needles from acetone-methanol

mp 118°

Solubility: most fat solvents very soluble
water insoluble

Stability: forms a molecular compound with calciferol, mp 122°

Preparation: by UV irradiation of a benzene-alcohol solution of ergosterol

Absorption max. 265 and 280 nm

Optical rotations: $[\alpha]_D^{19}$ in acetone +191.5°
$[\alpha]_{546}^{19}$ 2% in acetone +235.4°

Salts of lumisterol:

acetate: mp 100°

Optical rotations: $[\alpha]_D^{19}$ +130.5°
$[\alpha]_{546}^{19}$ 1.8% in acetone +163°

3,5-dinitrobenzoate: mp 139-141

Optical rotation $[\alpha]_{546}^{20}$ 1% in benzene +24°

allophanate: dec 217-218°

Optical rotation $[\alpha]_D$ in chloroform +75°

Stigmasterol:

mp 170°

Specific rotation -46°, -49°, or -51° by different authors (0071)

7-Dehydrostigmasterol:

Crystals

mp 154°

Solubility: in fat solvents very soluble

in water insoluble

Optical rotation: $[\alpha]_D$ in benzene -113.1°

Sitosterol: no data were found

7-Dehydrositosterol:

Platelets to ethanol

mp 144-145°

Solubility: in most fat solvents very soluble

in water insoluble

Stability: in air, browns on contact

Absorption max. in ethanol: 262, 271, 282, 293 nm

Optical rotation: $[\alpha]_D^{21}$ in chloroform -116°

Epi-7-dehydrocholesterol:

Crystals

mp 124-126°

Solubility: in most fat solvents very soluble

water insoluble

Optical rotation: $[\alpha]_D$ in chloroform -70.5°

7-Dehydrocholesterol:

Crystals

mp 152-153°

Solubility: in most fat solvents very soluble

water insoluble

Precipitated by digitonin

Absorption max in ethyl ether 280 nm

Optical rotation: $[\alpha]_D^{20}$ in chloroform -113°

Ergosterol:

Small white plates from ethanol

mp 168°

Solubility: in most fat solvents very soluble

water insoluble (and see Table 3)

Stability: to UV light destroyed

to oxidising agents decomposes

Absorption max in ethyl ether 280 nm

Optical rotation: $[\alpha]_D$ in chloroform -130° (see Table 4)

22,23-Dihydroergosterol:

Solvated needles from ethyl acetate and methanol

mp 152-153°

Solubility: in most fat solvents very soluble

water insoluble

Optical rotation: $[\alpha]_D^{19}$ in chloroform -109°

Brassicasterol:

mp 148°

Specific rotation -64°

Campesterol:

mp 158°

Specific rotation -33°

7-Dehydrocampesterol: no data were found

Fucosterol:

mp 124°

Specific rotations -38° and -40° by different authors (0071)

Epiergosterol: no data were found

22,23-Oxidoergosterol: no data were found

22-Dihydroergosterol (sic): (interpreted as 22,23-dihydroergosterol)

mp 153°

Specific rotation -109° (0071)

7-Dehydroclionasterol:

mp 138°

Specific rotation -98° (0071)

$\Delta^{5,7,22}$-Cholestatriene-3-ol: no data were given (0071)

$\Delta^{5,7}$-Norcholestadiene-3-ol: no data were found

3,17-Dihydroxyandrostanediene: no data were found

Table 3

Solubilities of Ergosterol (5204)

A. Data from Tanret

Solvent	Temperature, °C	Parts of solvent to dissolve 1 part
Acetone	20	200
Acetone	Boiling	32
Alcohol, 95%	Cold	526
Alcohol, 95%	Boiling	36
"Benzine"	16	94
Chloroform	18	50
Chloroform	Hot	Few
Ether, anhydrous	20	50
Ether, anhydrous	Boiling	28
Ether, hydrated	20	112
Ether, hydrated	Boiling	50
Water		Insol.

B. Data from Honeywell and Bills

Solvent	Temperature, °C	Milliliters of boiling solvent to dissolve 1 g.
Acetone	56	27
Alcohol, 96%	78	50
Benzene	80	4.6
Ether, U.S.P.	35	70
Ethyl acetate	77	6.5
Hexane	65-70	24
Isopropyl alcohol	82	10
Methanol	65	280
Methyl acetate	54	35
Methylcyclohexane	101	<2

Table 4

Optical Rotation of Ergosterol* (5204)

Solvent	$[\alpha]^{20}_{5461}$	$[\alpha]^{20}_{D}$	Ratio
Chloroform	-158.5°	-125.25°	1.27
Benzene	-156.0°	-124.0°	1.26
Ethyl acetate	-120.0°	- 95.0°	1.26
Ether	-120.0°	- 94.0°	1.27
Alcohol (absolute)	-119.0°	- 93.0°	1.28
Acetone	-118.0°	- 92.0°	1.28
	Average value of ratio =		1.27

*Data from Bacharach et al. on a commercial grade of ergosterol

Table 5

Optical Rotation of Calciferol* (5204)

Solvent	$[\alpha]^{20}_{5461}$	$[\alpha]^{20}_{D}$	Ratio
Alcohol (absolute)	**+125.0°**	**+106.25°**	**1.18**
Ethyl acetate	+113.25°	+ 95.0°	1.19
Ether	+105.5°	+ 88.75°	1.19
Benzene	+102.12°	+ 87.5°	1.17
Acetone	+ 99.5°	+ 83.5°	1.19
n-Hexane	+ 66.5°	+ 56.25°	1.18
Chloroform	+ 61.75°	+ 52.25°	1.18
	Average value of ratio =		1.18

*Data from Bacharach et al. on commercial lots of refined material

25-Hydroxyvitamin D_2:

λ max in ether 264 nm (ε587)

25-Hydroxyvitamin D_3:

λ max 265 nm (0630); nmr spectra, see fig. 1 (0630); for ms, see fig. 1 (0630) and 2 (4302).

1,25-Dihydroxyvitamin D_2: no data were found.

1,25-Dihydroxyvitamin D_3:

for ms, see fig. 2 (4302).

1α,25-Dihydroxyvitamin D_2: no data were found.

1α,25-Dihydroxyvitamin D_3: no data were found.

25,26-Dihydroxyvitamin D_3:

λ max in ethanol 265 nm (5586).

1α-Hydroxyvitamin D_3:

λ max 265 nm, m in 228 nm (2688).

5,6-*trans*-Vitamin D_3: no data were found.

Isovitamin D_3:

Absorption max 278, 288, 300 nm (2689).

mass spectrum, see fig. 3.

Isotachysterol:

Absorption max 280,290,302 nm in diethylether (2689).

mass spectrum, see fig. 3.

25-Hydroxytachysterol: no data were found.

VII. Analytical Methods

1. In 1934 the USP standardized the measurements for vitamin D activity (4212). In 1938 Nelson (4212) remarked that bioassays needed only one microgram of pure D, and practical chemical assays were limited to colorimetry. He commented that since D was one of "the ubiquitous sterols, the probability of developing color tests with a high degree of specificity seems rather remote."

From time to time modifications of USP methods or new methods have been proposed. Some examples are given.

2. In 1954 Ewing *et al.* (1722) described a two-step chromatographic method of separating vitamins D from A in nonsaponifiable oil fractions, using an activated earth and an activated alumina. Eluted D was measured at 265 nm, and data were within 20% of those obtained by bioassay.

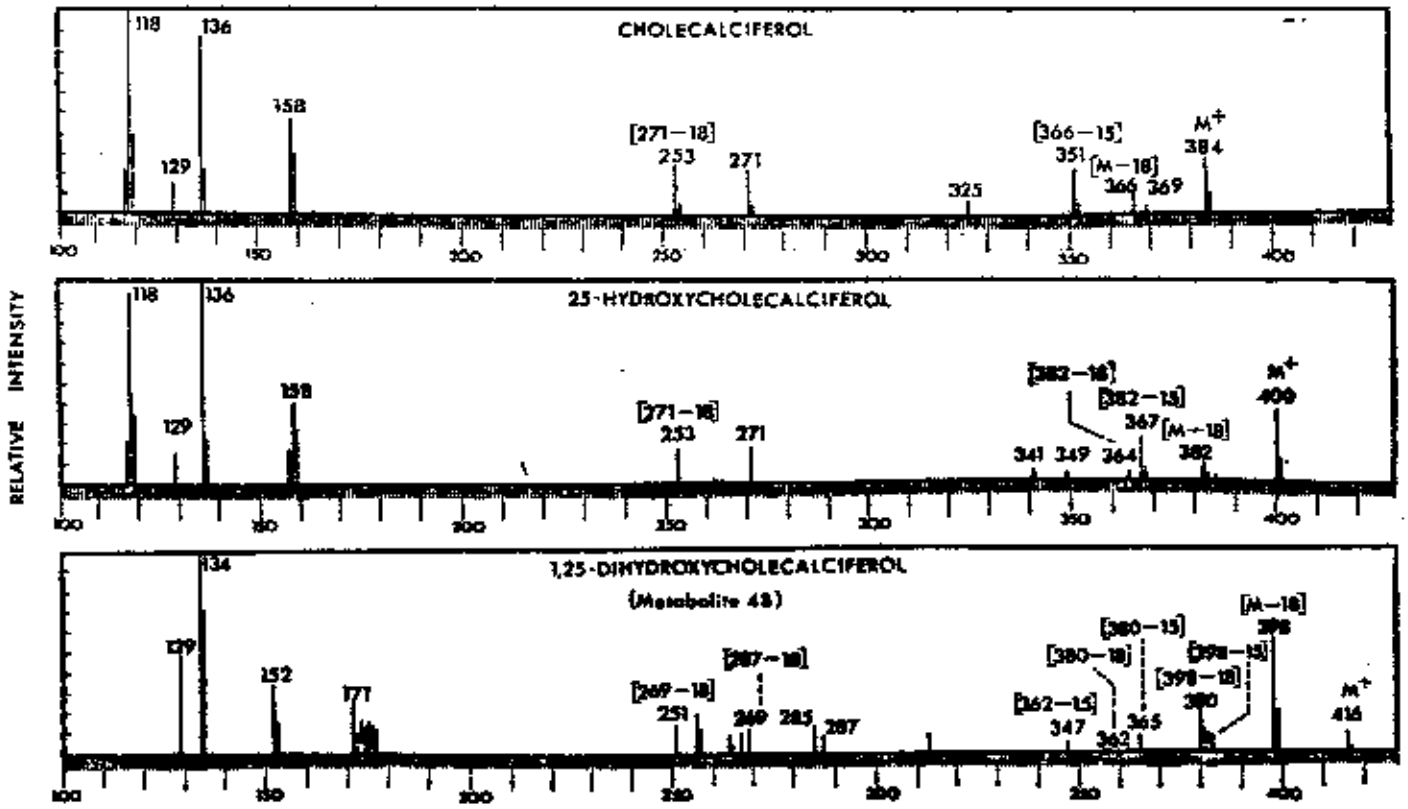


Fig. 2. Mass spectra of cholecalciferol, 25-hydroxycholecalciferol, and 1, 25-dihydroxycholecalciferol. (4302)

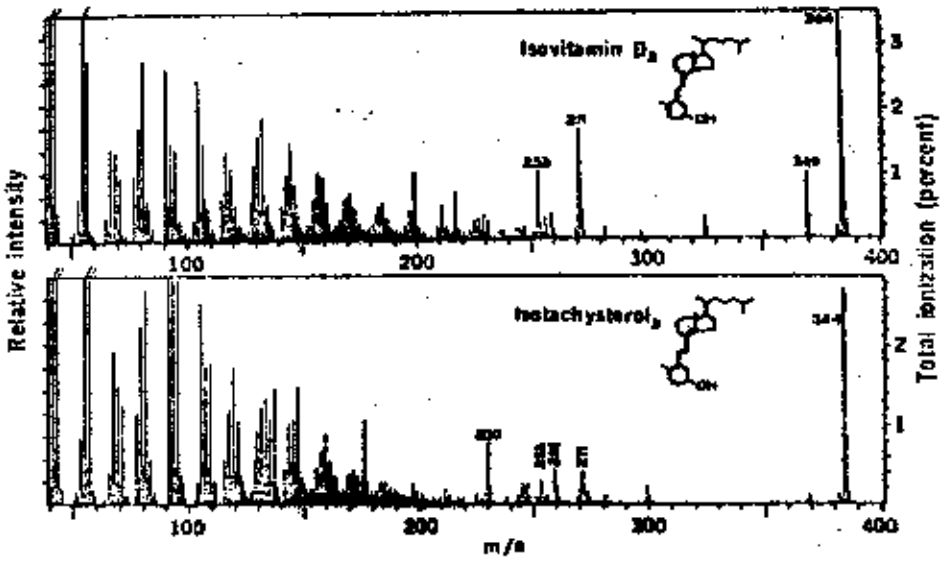


Fig. 3. Mass spectra of isovitamin D_3 and isotachysterol$_3$. (2689)

3. In 1955 Ewing et al. (1722) claimed an improvement in the separation of vitamins D_2 and A. This involved two-stage filtration (activated earth, activated alumina) followed by spectrophotometry at 265 nm, the absorption max of D_2.

4. In 1955 Numerof et al. (4329) reported that after Mills and others had, from 1931 to 1947, criticized the USP line test as too subjective for proper assay of D activity, Snyder, Eisner and Steenbock in 1951 claimed accuracy for an assay in which rats were primed with P^{32}. Numerof et al. found that the P^{32} method was superior to the line test but was reliable within narrower limits than had been claimed. The authors commented that bioassays for D activity remained superior to physiochemical assays.

5. In 1956 Kodicek (3208) noted in a review the following methods of assay for D:

The rat line test
The rat paw P^{32} test
The chick bone ash Ca^{45} test
Adducts of D_2 with benzoquinone, by titration
Spectrophotometry

6. In 1959 a posthumous report by Nelson, issued by the Council on Foods and Nutrition of the American Medical Association (4218), noted that D deteriorated rapidly on mixture with Ca salts, and that a new coated formulation of D had been claimed to resist the action of the Ca salts. The author recommended that the situation be monitored by use of a new chemical determination of D activity, published by Wilkie, Jones and Kline (J.Am.Pharm.A., Scient.Ed. 48:358-394, 1958).

7. In 1961 Bro-Rasmussen and Hjarde (0751) reviewed the assays for D and concluded that low-potency preparations could not be purified enough for assay by spectrophotometry or colorimetry. However, Sweeley and Horning in 1960 (reference cited) had separated steroids by gas chromatography, and this might be applied to the D vitamins. In Norway, official assays were still limited to bioassays.

8. In 1965, noting that the USP XVI Edition had specified for vitamin D chemical assay, a blank that was read at 500 nm, Shue (5327) proposed that reading at 550 nm would be more accurate, though not completely accurate. He stated that the calculations would need revision, but a simplified procedure would take samples of 5 μg containing 200 USP units.

9. In 1965 Pasalis and Bell (4451) reported that vitamin D esters could be separated by thin-layer chromatography using silica gel; the longer the chain, and the higher the saturation, the greater was the mobility.

10. In 1966 Jones and Libby (2993) modified the Jones assay for D in evaporated milk, by adding to the column steps a third column, of aluminum oxide.

11. In 1968 Blunt and DeLuca (0630) synthesized cholesta-5,7-diene-3β,25-diol by two different methods and converted it to 25-OH-D_3, which was positively identified by UV spectrophotometry, gas-liquid partition chromatography, nmr and high-resolution mass spectra. Their uses of these methods were fully described.

12. In 1968 Oesdce and DeRitter (4398) noted that the USP XVII Edition of 1965 method of D assay grossly overestimated the amounts of D in multi-vitamin preparations when tocopherols were present. The authors claimed that filtration through activated earth and alumina was incomplete, and proposed a removal of tocopherols by column chromatography using Mg phosphates.

13. In 1968 Brubacher (0783) detailed the USP XVI chemical assay for D, which is cited here (to be read in the original) because it is set out in an easily readable format.

14. In 1969 Eisses and DeVries (1651) modified the Jones colorimetric assay for D (USP Ed.XVI) by:

(1) eliminating "nearly all" cholesterol by digitonin precipitation;

(2) replacing the Florex column with an alumina column.

The authors claimed recovery of D at 78 ± 6 (SD) %.

15. In 1971 Norman et al. (4302) reported the mass spectra of D_3, 25-OH-D_3, and 1,25-$(OH)_2$-D_3, shown in Fig. 2 , and described their methodology.

16. In 1973 Holick et al. (2688) demonstrated the identity of 1α-OH-D_3, a synthetic analog that they had prepared from cholesterol, by its UV absorption spectrum, mass spectrum, and gas-liquid chromatogram.

17. In 1973 Yang et al. (6308) used young turkeys to bioassay 26 vitamin D_3 supplements, using bone ash as a response criterion. Another study of 22 supplements used toe ash. Growth, bone length, strength, rigidity, plasma APase and P_1, all correlated well with the ash criterion but not with the USP chemical assay criterion.

The authors concluded that the USP chemical assay measured biologically inactive forms of D as well as active forms, and that bone ash gave "the greatest assay precision."

18. In 1974 Brumbaugh et al. (0812, 0813) developed a competitive protein binding assay for 1α,25-$(OH)_2$-D_3 in human serum, using tissues isolated from chick small intestine.

VIII. Occurrence

1. In 1938 Nelson (4212) stated that there was "no proof" that living plant tissues contained D, although some dead tissues such as hay could acquire D activity by solar irradiation. Natural sources were limited to animal foods such as fish, eggs, and milk, and only milk need be considered seriously.

2. In 1940 Warkany and Nelson (6108) studied the vitamin D contents of human serum samples, using the line test in rats and a factor of 3.3 to convert Steenbock units into USP units. From 155 samples they obtained 89 "complete" assays, 30 from White adults, 34 from White children, and 25 from Negro children. The findings are shown in Table 6. The authors found no seasonal variations.

Table 6

Frequency Distribution of Persons According to Levels of Vitamin D in Blood Serum (6108)

U.S.P. Units per 100 CC. of Blood Serum	Total No. Tested	White Adults	White Children	Negro Children
165	9	2	6	1
132	27	10	11	6
110	31	14	10	7
94	8	2	4	2
83	13	1	3	9
66	1	1	0	0
Total	89	30	34	25
Average vitamin D level, units per 100 cc	116.4	117.6	122.6	106.5

The results were compared with D levels in other materials: cod liver oil 10,000-15,000 USP units/100 g, egg yolk 140-390 (seasonal), butter 120, mammalian liver 10-50, and cows' milk 0.5-4.0 USP units/100 ml (seasonal).

The authors commented that the overall average serum value from their tests was 110 USP units/100 ml, range 66-165 considered normal, and that the values in White children were 15% above those in Negro children.

4. In 1954 Sabrell and Harris (5204) listed the then known occurrence of the provitamins D in plants and animals (Table 7), the distribution of vitamin D in fish liver oils (Table 8), various measures of the potency of vitamin D in cod liver oil (Table 10), and the occurrence of steroids related to vitamin D in shellfish (Table 9).

Table 7

Occurrence of the Provitamins D in Plants and Animals[a] (520)

Source	Provitamin D, parts per thousand of total sterol
Phanerogams	
Cottonseed oil	28*
Rye grass	15
Scopolia root	14
Spinach	10
Wheat germ oil	10*
Cocksfoot grass	8
Horse chestnut	8
Rutabaga	2.8
Carrot	1.7
Bean	1.0
Cabbage	0.5
Cryptogams	
Mold, *Aspergillus niger*	*1000
Mushroom, *Cortinellus shiitake*	*1000
Ergot, *Claviceps purpurea*	900
Yeast, *Saccharomyces cerevisiae*	300*
Mold *Penicillium puberulum*	280
Alga, seaweed, *Fucus vesiculosus*	0.9
Vertebrates	
Skin, pig	46*
Skin, chicken feet	25*
Skin, rat	19*
Skin, wild pig	16
Skin, mouse	9
Skin, calf	7
Skin, human adult	4.2
Skin, human infant	1.5
Skin, cow	1.8
Skin, deer	1.6
Skin, eel	1.2
Skin, chicken trunk	0.06*
Liver, tuna, Japanese	11
Liver cod Atlantic	4.4
Liver, shark	1.0
Liver tuna	1.0
Liver, pig	1.0
Liver, cod, Japanese	0.99
Liver, halibut	0.60*
Liver tuna, bluefin	0.40
Liver whale	0.12
Brain, rabbit	0.70
Brain, lumpfish	0.59
Brain, human fetus	0.50*
Brain, sheep	0.40
Brain, deer	0.33

Table 7 (cont.)

Source	Provitamin D, parts per thousand of total sterol
Vertebrates - cont.	
Brain, human infant	0.20*
Brain, horse	0.19
Brain, cow	0.13*
Brain, human adult	0.06*
Eggs, duck, Chinese	60*
Eggs, duck, Dutch	13
Eggs, cod (roe)	5.5
Eggs, herring (roe)	2.4*
Eggs, hen	1.6*
Eggs, cormorant	1.0
Eggs, mire crow	1.0
Eggs, silver gull	1.0
Eggs, lumpfish (roe)	0.3
Body of frog	8.8
Venom of toad	4.6*
Wool fat, sheep	3.9*
Milk, cow	2.3
Placenta, cow	1.8
Pancreas, beef	1.8
Blood serum, cow	1.5
Spinal cord, beef	1.2
Mice gutted carcasses	0.80
Colostrum, cow	0.70
Thymus, cow	0.70
Bile, ox	0.50
Herring oil	0.50
Spleen cow	0.45
Milt, herring	0.40
Heart, calf	0.32
Lymph, dog	0.30
Rats, gutted carcasses	0.30
Gallstones, man	0.25*
Lung, calf	0.25
Blood, dog	0.10
Sclerotic aortas, man	Nil
Invertebrates	
Poriferans	
Commercial species of sponges	20
Cliona celata, sponge	<10
Spheciospongia vesparia, loggerhead sponge	<10
Halychondria panicea, sponge	6
Coelenterates	
Metridium dianthus, sea clove	86
Actinoloba dianthus, sea anemone	52*
Actinia equina, sea anemone	50
Urticina crassicornis, sea anemone	45

Table 7 (cont.)

Source	Provitamin D, parts per thousand of total sterol
Invertebrates - cont.	
Pennatula quadrangularis, sea pen	42
Alcyonium digitalum, sea finger	34
Anemonia sulcata, sea anemone	17
Commercial species of corals	1[illegible]
Australian species of sea anemones	[illegible]
Unidentified species of sea anemones	trace
Bryozoan	
Flustra securifrons, sea mat	[illegible]*
Annelids	
Tubifex (Sp.), waterworm	210*
Lumbricus terrestris, earthworm	170*
Arenicola marina, lugworm, sandworm	55*
Hirudo medicinalis, leech	39*
Nereis virius, ragworm	16*
Arthropods	
Tenebrio molitor, mealworm	120*
Melolontha vulgaris, cockchafer grubs	89
Gyronomus (Sp.), gnat	61
Dytiscus marginalis, diving beetle	60
Caraucius morosus, locust eggs	25
Aleurobius farinae, meal mite	25
Eriocheir sinensis, wool hand crab	23*
Blatta orientalis, cockroach	23
'Kiefernspinnerraupe,' pine caterpillar	22
Cantharis vesicatoria, spanish fly	22
Cancer pagurus, common crab	15*
Bombyx mori, silkworm eggs	8.9*
Daphnia (Sp.), waterflea	7.5
Musca domestica, housefly	7.0
Melolontha vulgaris, cockchafer, May beetle	5.0
Apis mellifica, honeybee	4.5
Crangon vulgaris, shrimp	3.8*
Munida barffica, crustacean	3.5
Homarus vulgaris, lobster	2.5
Mollusks	
Modiolus demissus, ribbed mussel	370*
Arion empiricorum, slug, red road snail	220*
Buccinum undatum, whelk, wave horn snail	180*
Littorina littorea, periwinkle	170*
Archidoris tuberculata, sea snail	150
Ostrea (Sp.), Australian oyster	130
Arion (Sp.), black road snail	120
Mytilus edulis, sea mussel	100*
Helix pomatia, edible snail, vineyard snail	97*
Anodonta cygnea, swan mussel	80
Ostrea virginica, oyster	80
Pecten (Sp.), Australian scallop	65

Table 7 (cont.)

Source	Provitamin D, parts per thousand of total sterol
Invertebrates - cont.	
Mytilus planulatus, Australian mussel	62*
Cardium edule, cockle, sand shell	50*
Cardium tenuicostatum, Australian cockle	41*
Ostrea edulis, oyster	34*
Limax agrestis, earth snail	32
Sepia (Sp.), cuttlefish, squid	12*
Echinoderms	
Astropecten irregularis, little sea aster	4.5
Asterias rubens, starfish, big sea aster	3.8

[a]Data from Windaus, Gillam and Heilbron, van derVliet, and others. Figures marked (*) are the average of two or more determinations.

Table 9

Distribution of Vitamins D and A in the Liver Oils of 100 Species of Fish[a] (5204)

Common name of fish	Scientific name	Zoological order	Vitamin D, I.U./g	Vitamin A, I.U./g
Oriental tuna*	*Thunnus orientalis*	Percomorphi	45,000	170,000
Frigate mackerel*	*Auxis thazard*	Percomorphi	44,000	30,000
California bluefin tuna*	*Thunnus saliens*	Percomorphi	42,000	65,000
Striped tuna*	*Katsuwonus pelamis*	Percomorphi	42,000	36,000
Meji tuna*	*Parathunnus sibi*	Percomorphi	38,000	22,000
Bonito*	*Sarda lineolata*	Percomorphi	35,000	57,000
Yellowtail*	*Seriola dorsalis*	Percomorphi	25,000	67,000
Red snapper	*Lutianus camechanus*	Percomorphi	22,000	61,000
Atlantic tuna	*Thunnus secundodorsalis*	Percomorphi	16,000	80,000
Albacore*	*Thunnus germo*	Percomorphi	13,000	12,000
Yellowfin tuna*	*Neothunnus macropterus*	Percomorphi	12,000	35,000
White sea-bass*	*Cynoscion nobilis*	Percomorphi	11,000	92,000
Jewfish*	*Stereolepis gigas*	Percomorphi	9,000	500,000
Ishinagi*	*Stereolepis ishinagi*	Percomorphi	7,000	500,000
Swordfish*	*Xiphius gladius*	Percomorphi	7,000	130,000
Black perch	*Embiotoca jacksoni*	Holconoti	7,000	6,000
Broadfin sole	*Lepidopsetta bilineata*	Heterosomata	6,800	13,000
Oriental mackerel*	*Scomber japonicus*	Percomorphi	6,300	59,000
Corsair rockfish	*Sebastomus rosaceus*	Cataphracti	5,800	75,000
Mackerel scads	*Decapturus muroadsi*	Percomorphi	5,800	5,900
Grouper	*Epinephelus morio*	Percomorphi	4,800	24,000
Barracuda*	*Sphyraena argentea*	Percomorphi	4,700	67,000
Starry rockfish	*Sebastomus constellatus*	Cataphracti	4,500	89,000
Yellowtail rockfish	*Sebastosomus flavidus*	Cataphracti	4,000	92,000
Red rockfish	*Sebicola miniatus*	Cataphracti	3,600	14,000
Totuava*	*Erisecion macdonaldi*	Percomorphi	3,500	190,000
Jack smelt	*Atherinopsis californiensis*	Percomorphi	3,400	96,000
Spearfish*	*Makaira mitsukurii*	Percomorphi	3,300	120,000
Bastard halibut*	*Paralichthys californicus*	Heterosomata	2,300	60,000
Sardine (pilchard)	*Sardinia caerulea*	Isospondyli	2,300	16,000
Rockfish	*Pteropodus vexillaris*	Cataphracti	2,200	49,000
Red rockfish	*Sebastopyr ruberrimus*	Cataphracti	2,100	100,000
Snook*	*Thyrietes atun*	Percomorphi	2,000	33,000
Bocaccio	*Sebastodes paucispinis*	Cataphracti	1,800	77,000

Table 8 (cont.)

Common name of fish	Scientific name	Zoological order	Vitamin D, I.U./g	Vitamin A, I.U./g
Yellowbacked rockfish	*Pteropodus maliger*	Cataphracti	1,800	32,000
Pacific hake	*Merluccius productus*	Anacanthini	1,500	50,000
Black rockfish	*Sebastosomus mystinus*	Cataphracti	1,500	37,000
China rockfish	*Pteropodus nebulosus*	Cataphracti	1,400	110,000
Fringe sole	*Psettichthyl melanostictus*	Heterosomata	1,400	10,000
Halibut*	*Hippoglossus hippoglossus*	Heterosomata	1,200	75,000
Shad	*Alosa sapidissima*	Isospondyli	1,200	17,000
Striped bass	*Roccus saxatilis*	Percomorphi	1,200	4,500
Orange rockfish	*Rosicola pinniger*	Cataphracti	1,100	86,000
Green spotted rockfish	*Sebastomus chlorostictus*	Cataphracti	1,100	47,000
Rockfish	*Acutomentum entomelas*	Cataphracti	1,100	8,000
Wall-eyed perch	*Hyperprospon argenteus*	Holconoti	1,100	3,500
Rabbitfish	*Cyclichthyl schaepfi (?)*	Plectonathi	1,100	2,200
Starry flounder	*Platichthys stellatus*	Heterosomata	1,000	8,200
Striped rockfish	*Hispaniscus elongatus*	Cataphracti	990	74,000
Ainame	*Hexagrammos otakaii*	Cataphracti	950	3,900
Ling cod*	*Ophiodon elongatus*	Cataphracti	920	160,000
Striped perch	*Taeniotoca lateralis*	Holconoti	900	4,300
Rubberlip perch	*Rhacochilus toxotes*	Holconoti	890	3,300
Rockfish, black bass	*Sebastosomus melanops*	Cataphracti	830	40,000
Spanish flag rockfish	*Hispaniscus rubrivinctus*	Cataphracti	810	32,000
Boston mackerel	*Scomber scombrus*	Percomorphi	750	31,000
California mackerel*	*Pacumatophorus diego*	Percomorphi	730	45,000
Pufferfish	*Sphaeroides maculatus*	Plectonathi	570	1,500
Cabezon	*Scorpaenichthyl marmoratus*	Cataphracti	530	16,000
Round-nose sole*	*Eopsetta jordani*	Heterosomata	520	76,000
Nibo	*Sciaena mitsukurii*	Percomorphi	500	5,400
Pacific white perch	*Phanerodon furcatus*	Holconoti	460	6,400
Fork-tail perch	*Damatichthys argyrosomus*	Holconoti	410	2,700
Black cod*	*Anoplopoma fimbria*	Cataphracti	310	42,000
Chili-pepper	*Sebastodes goodei*	Cataphracti	270	150,000
Cabrilla*	*Epinephelus analogus*	Percomorphi	260	160,000
Newfoundland turbot	*Reinhardtius hippoglossoides*	Heterosomata	260	7,000
Pacific cod*	*Gadus macrocephalus*	Anacanthini	190	4,800

Table 8 (cont.)

Common name of fish	Scientific name	Zoological order	Vitamin D, I.U./g	Vitamin A, I.U./g
Atlantic salmon	*Salmo salar*	Isospondyli	180	12,000
Rock cod	*Sebastolobus alascanus*	Cataphracti	150	6,400
Rex sole	*Errex zachirus*	Heterosomata	140	8,200
Pointed sole	*Parophrys vetulus*	Heterosomata	140	6,100
Soulpin	*Scorpaena guttata*	Cataphracti	140	3,000
Sand dab	*Citharichthys sordida*	Heterosomata	120	3,700
Atlantic hake*	*Urophycis* (Sp.)	Anacanthini	120	2,300
California turbot	*Pleuronichthys decurrens*	Heterosomata	110	8,200
Menuke*	*Sebastodes baramenuke*	Cataphracti	100	120,000
Atlantic cod*	*Gadus morrhua*	Anacanthini	100	1,400
Yellow sole	*Pseudopleuronectes dignabilis*	Heterosomata	87	17,000
Widowfish	*Aculomentum ovale*	Cataphracti	82	73,000
Tinker mackerel	*Pneumatophorus grex*	Percomorphi	77	9,300
Atlantic pollack*	*Pollachius virens*	Anacanthini	70	2,300
Pacific pollack*	*Theragra chalcogramma*	Anacanthini	67	8,300
Sheepshead	*Pimelometopon pulcher*	Pharyngognathi	62	6,6000
California flying fish	*Cypselurus californicus*	Synentognathi	51	35,000
Corbina	*Ventricirrhus undulatus*	Percomorphi	51	11,000
Rosefish*	*Sebastes marinus*	Cataphracti	33	26,000
Common skate of California	*Raja inornata*	Batoidei	25	9,800
Abura karei	*Hippoglossoides dubius*	Heterosomata	25	5,100
Big skate of California*	*Raja binoculata*	Batoidei	24	4,100
Kichiji	*Sebastolobus macrochir*	Cataphracti	22	4,900
Wolffish	*Anarhichas lupus*	Jugulares	19	1,300
Pacific dogfish*	*Squalus suckleyi*	Tectospondyli	13	13,000
Same karei	*Clidoderma asperrimum*	Heterosomata	12	4,900
Thresher shark	*Alopias vulpinus*	Euselachii	9	2,400
Basking shark	*Cetorhinus maximum*	Euselachii	6	<100
Atlantic dogfish	*Squalus acanthias*	Tectospondyli	3	1,700
Ratfish	*Hydrolagus colliei*	Chimeroidei	2	180
Gray sole	*Glyptocephalus cynoglossus*	Heterosomata	<2	8,900
Sturgeon	*Acipenser fulvescens*	Glanostomi	<1	600

[a]From Bills et al. with revisions; nomenclature from Jordan et al. wherever possible. The species marked (*) are represented by more than one assay.

Table 9

Sterols of Molluska (Bergmann) (5204)

Class and species	Melting point of steryl acetate, °C	Principal sterol
Pelecypoda		
Tapes phillippinarum	137	
Corbicula leana	126-127	Corbi-, brassicasterol
Cristaria plicata	137-138	
Meretrix meretrix	137-138	Meretristerol
Ostrea gigas	136-137	Conchasterol
Ostrea virginica	134-135	Ostreasterol
Mya arenaria	131	
Volsella modiolus	127-128	
Tridacna gigas	156-157	Shakesterol
Venus mercenaria	131	
Modiolus modiolus	131	
Modiolus demissus	156-157	Brassicasterol
Gastropoda		
Haliotis gigantea	117	Cholesterol
Turbo cornutus	116	Cholesterol
Rapana thomasiana	120	Cholesterol
Collana nigrolineata	115	Cholesterol
Tegula xanthostigma	118	Cholesterol
Fulgur (Sp.)	117	Cholesterol
Buccinum undatum		Cholesterol
Littorina littorea		Cholesterol
Nerita peleronta		Cholesterol
Nassa obsoleta		Cholesterol
Cephalopoda		
Sepia officinalis		Cholesterol
Octopus vulgaris		Cholesterol

Table 10

Vitamin D Content of Average Cod Liver Oil in Terms of Various Systems of Units. (Adapted from Bills) (5204)

Unit system	Potency
International, since 1931 (see text)	100 units/g
U.S.P., since 1934 (see text)	100 units/g
Medical Research Council, 1930	100 units/cc
Steenbock, 1930	37 units/g
American Drug Manufacturers' Association, 1931	350 units/g[a]
Oslo, 1928 (Poulsson and Lovenskiold)	110 units/g[a]
Oslo, 1933 (Poulsson and Ender)	160 units/g[a]
German, 1939 (rat unit)	15 units/cc
German (clinical unit)	0.15 units/cc
American Medical Association, 1931	2.8 "D" potency

[a]Vaguely defined.

5. In 1961 Bro-Rasmussen and Hjarde (0751) cited the following calculations of rates of D synthesis in the skin, induced by UV:

a. In pigs, approximately 30-100 IU/cm^2 (Bekemeier and Pfenningsdorf, Z. Physiol. Chem. 314:120, 1959.)

b. In rats, 5-15 IU/cm^2 (Bekemeier and Pfenningsdorf, Z. Physiol. Chem. 314:120, 1959.)

c. In man, 4-18 IU/cm^2 in 3 hours (Bekemeier, Acta Biol. Med., 1:756-757, 1959.)

d. About 15-30% of the provitamin present was activated, according to Bekemeier and Pfenningsdorf, in the reference above cited.

e. In rats after removal of the fur, UV produced about 290 IU of D activity, according to Cruikshank and Kodicek, in Proc. Nutr. Soc. 14:viii, 1955.

6. Using the Bekemeier rate (1958, in 0751 above) Loomis calculated in 1967 (3575) that 20 cm^2 of an infant's cheeks would synthesize about 400 IU per day by daily exposure outdoors.

He cited work by M.L. Thomson (J. Physiol. Lond. 127:236, 1955) showing that sunshine of 300-400 nm (290-320 nm being photosynthetic for D) penetrated the isolated stratum corneum in different amounts for Europeans and Africans. In Africans 18 (3-36) percent of the light penetrated the specimens, in Europeans 64 (53-72) percent. In an albino African 53% penetrated. Loomis calculated that in the tropics a European could synthesize up to 800,000 IU/day, but a deeply pigmented African 4,000-8,000 IU/day. In his opinion the latter range was acceptable, the former pathogenic. He contended that subsequent controversy did not invalidate his calculations (3576).

7. In 1969 Ponchon and DeLuca (4603) injected 10 IU of 1,2-^{3}H-D$_3$ into D-deficient rats and isolated eleven radioactive metabolites. One, 23-OH-D$_3$, had "intense" antirachitic activity, and the others were not identified.

The *p*-nitrobenzoate, 3,5-dinitrobenzoate, phenylurethan, allophanate and other listed salts of the D vitamins are laboratory preparations of natural extracts (5511).

8. In 1972 Altman and Dittmer (0071) documented the occurrences of a number of naturally occurring sterols:

Stigmasterol	Animals in general, rat adrenals, echinoderms, ant pharyngeal glands, earthworms, mollusks, protozoa, breast cancers, feces, plants in general, *E. coli*, tobacco, herbs, spices, bark, flowers, leaves, pollen, roots, seeds, tubers, wood, vegetable oils, and Mycota.
7-Dehydrostigmasterol	Tunicates, shellfish, protozoa, philodendrons.
7-Dehydrocholesterol	Human breast cancer, rats and their organs, swine skin, crustaceans, crickets, annelids, mollusks, protozoa, amniotic fluid, meconium, vernix caseosa, feces.
Ergosterol	Starfish, earthworms, snails, protozoa, schizomycetes, algae, mycota, molds, yeasts, basidiomycetes, mycelium.
22,23-Dihydroergosterol	Protozoa, chlorophyta, mycota, phycomycetes, Claviceps, basidiomycetes.
Brassicasterol	Fish, shellfish, echinoderms, crustaceans, earthworms, mollusks, coelenterates, protozoa, phaeophyta, rhodophyta, brassicaceae (cruciferae) radish seeds, wheat, rapeseed oil, vegetable oils.
Campesterol	Animals in general, rat adrenals, echinoderms, insects, earthworms, mollusks, breast cancer, feces, plankton, plants in general, *E. coli*, mycota, tobacco, bark, corms, fruits, grain, leaves, pollen, roots, seeds, tubers, wood, vegetable oils.
Fucosterol	Echinoderms, mollusks, phaeophyta, rhodophyta, phycomycetes, ferns, pea leaves, coconuts, pollen.
$5,7,22$-Cholestatriene-3-ol	Shellfish

9. In 1974 Wasserman (6122) isolated a factor from *Solanum malacoxylon*, from Argentina, that was potent for activity similar to that of $1,25\text{-}(OH)_2\text{-}D_3$ in cattle and chicks; he claimed that this was the first such factor isolated from a plant. Since it was water-soluble, the author concluded that the factor was unlikely to be a sterol; it was not yet characterized.

10. In 1974 Brumbaugh et al. (0812, 0813) developed a competitive protein binding assay for $1,25\text{-}(OH)_2\text{-}D_3$, using tissue from isolated chick small intestine, and calculated that normal human serum contained approximately 6 ng/100 ml of the hormonal form of D_3.

BIOLOGICAL DATA

I. Acute Toxicity

With vitamin D substances lethal toxicity is not always immediate. Some lethal toxicity data are therefore summarized here from studies reported in detail under short-term toxicity.

A. Mice

Sahashi et al. (4990) determined the LD_{50} of i.p. D_2 sulfate in mice (20 g) to be 2,500,000 IU/kg BW. The authors noted that the death rate for this compound was much lower than for free D_2. (Toxicity data for the ammonium and sodium salts of D_2 sulfate are shown in Table 11.)

B. Rats

1. Harris et al. (2438) fed rats 50,000 USP units/rat/day of either irradiated ergosterol or tuna liver oil. All lost weight and died between 17-31 days of feeding. (For experimental details see Biological Data II.)

2. McChesney (3821) found the equivalent toxic doses for rats (300-600 g) administered D_2, D_3 or dihydrotachysterol in corn oil by stomach tube to be respectively, 3.60 mg/kg/day, 2.30 mg/kg/day, and 1,000 mg/kg/day. These were calculated from the experimental data using a median 20-day survival time. (For experimental details see Biological Data II.)

C. Rabbits

1. Haas et al. (2499) administered 100,000 units viosterol bi-weekly for more than three weeks to male albino rabbits. Death occurred within about six weeks following anorexia and loss of weight. (For experimental details see Biological Data II.)

2. Matsuda and Kato (3770) administered s.c. D_2 daily to three groups of five rabbits each for three consecutive days in doses of 400,000 IU/kg, 100,000 IU/kg, and 10,000 IU/kg, respectively. Death occurred five to seven days after injection following anorexia and weight loss. (For experimental details see Biological Data II.)

3. Friedman and Roberts (1947) gave three groups of five adult female rabbits i.m. injections of 2.5, 3.5, and 4.5 million units (total amount) of activated ergosterol in cottonseed oil. All died within 65 days of their first injection.

Table 11

Toxicity of Vitamin D_2 Sulfate in Mice (4990)

Material	Injected dose[b,c]	Death rate after 24 hr[a]	Death rate after 48 hr[a]
	10^3IU(as vitamin D_2)		
Ammonium vitamin D_2 sulfate	2	0/10	0/10
	4	0/10	0/10
	6	0/10	0/10
	12	0/10	0/10
	30	0/10	0/10
	40	0/ 0	0/10
	45	4/10	6/10
	50	8/10	10/10
Sodium vitamin D_2 sulfate	2	0/10	0/10
	4	0/10	0/10
	6	0/10	0/10
	12	0/10	0/10
	30	0/10	0/10
	40	0/10	0/10
	45	4/10	6/10
	50	6/10	10/10

a The number of the dead mice/the number of mice used.

b The dose per 20 g of the body weight of the mice.

c Each volume injected per head was 0.5 ml of saline solution.

D. Dogs

1. Taylor et al. (5714) found a minimum lethal dose for irradiated ergosterol given to an adult female dog (16 kg) to be about 5.00 cc in five days. Death occurred on the fifth day. The average daily dose for the period was 0.068 cc/kg.

Another dog, a healthy male (30 lbs.) died after 84 hours when administered 0.15 cc/kg daily. The serum calcium before death was elevated to 19 mg per 100 cc. On the day following the first dose the animal became depressed, weak, and lethargic with reduced muscular tone. This was followed by diarrhea and vomiting with blood.

2. Taylor and Weld (5713) gave a young collie (3-4 months, 4 kg) 1 cc irradiated ergosterol per os followed three days later by 2 cc. Within a few hours there was extreme weakness and vomiting. Death occurred within 48 hours. The mucosa and stomach showed an extremely intense reaction.

Four young pups (2 months old) were given 0.5, 0.4, 0.3, 0.2 cc irradiated ergosterol per os on two successive days. The results are shown in Table 12.

3. Steck et al. (5512) gave adult dogs activated ergosterol in corn oil or calciferol in corn oil per os, 1,000,000 units per g. Some received the same materials i.v. in daily doses ranging from 15,000 to 500,000 units/kg BW. More than 20,000 units/kg/day was found to be fatal to 35 out of 43 animals.

E. Humans

1. Debré (1344) reported two fatal infant cases, one at 20 months administered 11,200,000 units D_2 and the other at 16 months administered 18,200,000 units D_2.

2. DeWind (1466) reported the case of a 5.5 year-old boy who had ingested large quantities of D (exact amount not stated) for one year causing a reversible increase in bone density, severe calcinosis and renal failure.

3. In 1966 Henkin reported at a symposium (0161, 2550) a case of a female aged 54, who died after receiving 100,000,000 IU D per os and i.m. over 3.5 months for D-resistant osteomalacia, resulting in serum levels of over 3500 IU/100 ml D and 20.4 mg/100 ml Ca, and a series of grand mal seizures. Peritoneal dialysis removed 27,000 IU D, reducing the serum levels to 1350 IU/100 ml with clinical improvement, and a second dialysis removed

Table 12

Effect of Two Large Doses of Irradiated Ergosterol (5512)

Pup No.	Weight in kilos.	Number of days after first dose till death.	Total dose (10,000 X) in c.c.	Calculated dose per day= total dose÷ days of survival.	Calculated dose per kilo per day in c.c.	Viosterol (250 D) equivalent per kilo per day in c.c.	X T*
110	1.46	7.5	1.0	0.13	0.08	8.0	66
111	1.39	8	0.8	0.10	0.07	7.0	58
112	0.91	5	0.6	0.12	0.13	13.0	108
113	1.6	8	0.4	0.05	0.03	3.0	25

T* = maximal therapeutic dose.

59,000 IU, but BUN continued to rise to 150 mg/100 ml, and she died. Removal of vitamin D failed on this occasion to influence the azotemia.

II. Short-Term Studies

A. In Vitro

In 1969 Eisenstein et al.(1650) studied the effects of sera of various compositions on rat arterial segments in vitro, isolated from general body metabolism. The rat arterial segments were incubated in several categories of rat serum:

a. from rats with hypervitaminosis D
b. from rachitic rats
c. from normal rats
d. from normal rats with added vitamin D
e. from normal rats with added calcium
f. from normal rats with both vitamin D and calcium added

It was observed that:

a. Serum from hypervitaminotic animals or normal sera enriched with both vitamin D and calcium induced increased accumulation by the arteries as early as 48 hours after incubation was started.
b. The amount of calcification was greatest in segments incubated in serum from hypervitaminotic D rats and the least in segments incubated in serum from rachitic rats.
c. Addition of either vitamin D or calcium alone to normal serum did not result in excess calcium accumulation in the incubated arteries.
d. The arteries showed a similar sequence of calcification in vitro and in vivo. Calcification began in the aortic arch, spreading centrifugally. The calcification was localized in the elastic tissue, the amount of elastic tissue in the artery paralleling the amount of calcium accumulation.

B. Rats

1. In 1929 Light et al.(3525) studied the effects of overdosage of rats for four weeks with 40 to 100,000 times the daily curative dose of the newly discovered irradiated ergosterol, later to be known as provitamin D_2. Four litters of six rats were divided into six groups of four rats each.

Four groups were fed stock diet and 40, 50, 1040, or 100,040 times the curative dose of D_2. Two groups were fed the Steenbock diet (Table 1) and 50 or 100,040 times the curative dose of D_2. Food offered, food not eaten, urine, feces, and bone were ashed, and serum Ca and P were determined.

This study and others cited in the paper or referred to as in progress led the authors to conclude that:

a. Doses up to 10,000 times the curative dose daily for six months did not noticeably affect growth or bodily functions of white rats.
b. Excessive amounts of D drained the body of minerals, with P losses relatively greater than Ca losses.
c. Doses of 100,000 times the daily curative dose produced anorexia, emaciation, greasy hair, labored breathing, and eventually death. Deaths occurred during the fourth week of the experiment.

2. In 1932, the Mead Johnson Symposium on The Present Status of the Knowledge of Vitamins (1064) reported experiments carried out by Light, Miller, and Frey of the Fleischmann Laboratories who studied rats for four generations fed the Steenbock diet 2965 and 40 units of D daily. They found no harmful effects in the first three generations. The fourth generation however, was more sensitive to overdosage than controls, and the harmful effects of larger daily doses which were just noticeable in the first generation were marked in the fourth. The authors concluded that smaller doses, just on the verge of toxicity in the first generation, may produce a cumulative effect in the third or fourth.

3. In 1939 Harris et al.(2438) determined the pathological effects of large quantities of vitamin D in the form of irradiated ergosterol or tuna liver oil and compared the relative toxicities of these two D sources to rats.

Four series of feeding experiments were carried out with month-old Wistar strain albino rats as follows:

Series 1: In this experiment the vitamin supplements were mixed with the diet. Two groups of five rats each were fed an equivalent of 16,000 USPXI units of D/rat/day for 20 days. The D was fed to one group in the form of tuna liver oil with a potency of 80,800 USP units/g and to the second group in the form of irradiated ergosterol with a potency of 7,273,000 USP units/g.

Series 2: The D supplements were also mixed in the diet in this experiment in which two groups of five rats each were fed an equivalent of 50,000 USP units

of D/rat daily until death. The potency of the tuna liver oil was the same as in the series 1 experiment but the potency of the irradiated ergosterol solution was 1,000,000 USP XI units/g.

Series 3: In this experiment as in series 2, each group of five rats was fed 50,000 USP units of one of the two same D supplements. However, this time the supplements were diluted with ethyl alcohol and one cc was delivered into the diet daily until death. The potency of the tuna liver oil concentrate used was 3,000,000 USP units/g.

Series 4: In this experiment, the two groups of five rats were each fed daily until death 80,000 USP units of one of the two D supplements (the feeding method was not stated). The potency of the tuna liver oil concentrate was 3,000,000 USP units/g and that of the irradiated ergosterol was 1,000,000 USP units/g.

The observations following histological examination were:

Series 1: No evidence of hypervitaminosis was found.

Series 2: All animals lost weight. Death occurred between day 17 and 31 of the supplement feeding period. Histological examination showed calcification of the kidney, aorta, stomach, lung and heart. No abnormality was noted in the spleen, liver and adrenal body. The effect of the irradiated ergosterol was greater than that of the tuna liver oil.

Series 3: All animals lost weight. Death occurred within about 13 days. The most severe calcification was found in the kidney. The ergosterol-fed group again showed heavier deposits.

Series 4: More weight was lost by these animals. They died sooner than the series 2 animals. Both groups, tuna liver oil, and irradiated ergosterol-fed, showed calcification of the kidney, stomach and heart. Only the ergosterol-fed group showed calcification of the aorta and lung. The calcification was again more severe in the ergosterol-fed group.

The authors concluded that:

a. Histologically detectable tissue changes were produced in the kidney, stomach, aorta, heart and lung of rats by daily oral feeding of 50,000 USP units of D either as tuna liver oil or irradiated ergosterol.

b. As measured by tissue pathology, the irradiated ergosterol was more toxic than the tuna liver oil. The calcification produced by daily

feeding of 50,000 USP units of irradiated ergosterol was more severe than that from 80,000 USP units of tuna liver oil concentrate.

c. The kidney, stomach, aorta, heart and lung were more readily calcified than the liver, spleen and adrenals.

4. In 1942 Lund and Armstrong (3609) examined the effect of a low calcium and vitamin D free diet on the composition of the bones and of the enamel and dentin of the molar and incisor teeth of rats.

Twenty male albino rats (11 months, 332 g.) were divided into two groups after five weeks on an adequate diet. One group, the controls, was continued on this diet for 220 days, the other was fed an experimental diet low in calcium and D for the same period of time.

It was observed that:

a. The calcium balance of the animals on the experimental diet was negative.

b. The molar teeth of the experimental rats crumbled easily but were not decalcified.

c. The alveolar bone was decalcified, the alveolar crest resorbed, and the teeth were loose in their sockets in the experimental animals.

d. Their incisor teeth were normal.

e. The average density of the humeri from the experimental animals was decreased, but their volume was unchanged which indicated a loss of bone substance from the interior but not from the surface of the bone.

5. In 1943 Reynolds and Burns (4808) did not find any histological or radiological changes in rats fed up to 20,000 IU/kg daily for varying periods of time. The treatment protocol is summarized in Table 13. At the end of various treatment periods animals were sacrificed to histologically examine their organs and tissues. X-ray studies were made of half the animals in each group.

A total of 47 male white rats (85 to 107 g) were divided into four groups of 10 animals each with seven animals as controls. Ertron, an electrically activated ergosterol dissolved in corn oil, was administered daily by pipette (see Table 13).

The observations were:

a. No changes were noted as compared to controls in the heart, lungs, liver, spleen, pancreas, stomach, adrenal glands, kidneys, aorta, and brain.

Table 13

Treatment Protocol for Administration of Ertron to Rats (4808)

Animal number	IU per Kg daily	Days of treatment
10, 11		100
12, 13, 14	4,000	127
15, 16, 17, 18, 19		190
20, 21		100
22, 23, 24	8,000	127
25, 26, 27, 28, 29		190
30, 31		100
32, 33, 34	12,000	127
35, 36, 37, 38, 39		190
40, 41		100
42, 43, 44	20,000	127
45, 46, 47, 48, 49		190
C1	0	100
C2, C3, C4	0	127
C5, C6, C7	0	190

b. No abnormality was noted in the bone tissues of either the treated or control animals.

c. No signs of alteration in bones, joints, cartilages or other structures were observed in the roentgenograms of the animals treated for 190 days as compared to controls.

d. None of the animals showed any signs of toxicity.

e. All remained in apparent good health until sacrifice.

The authors noted that their observations were based only on one particular D preparation, Ertron.

6. In 1943, Ziskin et al. (6376) studied the effect of massive daily doses of vitamin D_2 on dentin apposition, bone apposition, tooth eruption, calcification rhythm, skeletal calcification, pulp stone formation, estrus cycle and toxicity in rats.

Thirty-two female white rats (Sherman strain, 57 to 59 days old) were divided into two groups: 12 controls and 20 experimental animals. The experimental animals alternated a control period of two weeks with an experimental period of two weeks to allow the rat incisor to renew itself. The experimental animals each received 10,000 USP units of D (Ertron) incorporated in their diet. The daily dose was between 60,000 to 100,000 USP units/kg BW.

The observations were:

a. None of the animals showed grossly observable signs of toxicity or deleterious effects during the course of the experiment while being fed the vitamin preparation. The animals appeared healthy and their weight increased.

b. The treated animals showed an accelerated dentin apposition.

c. In the treated animals a marked increase in bone formations was seen during the experimental, as compared with the control, period (no D fed). The untreated rats (controls) showed no such increase.

d. There was no significant influence on the calcification period.

e. There was significant effect on the eruption rate.

f. No pulp stones were found.

g. The estrus cycle was unaltered during the experimental period as compared to the control period.

7. In 1944, McChesney (3821) carried out experiments to determine whether the toxicities and hypercalcemic effects in rats of crystalline D_2 and D_3 and dihydrotachysterol were related. Albino rats (225 to 275 days old, 300 to 600 g) were administered the medication tested in corn oil by stomach tube. Each rat received 0.125 cc per 100 g BW daily (0.1875 cc per 100 g on Saturday and Monday). The animals were sacrificed after 14 days for the hypercalcemic study. The results of the experiments are summarized in Tables 14 and 15.

The author concluded that in the albino rat:

a. Crystalline D_3 was 55% more toxic than dihydrotachysterol.

b. Dihydrotachysterol was about 260% more toxic than crystalline D_2.

c. The hypercalcemic effect of D_2 and D_3 correlated with their toxicities.

d. The hypercalcemic effect of dihydrotachysterol did not correlate with its toxicity.

8. In 1963, Murray and Beare (4141) investigated the possible relationship between D toxicity and linoleate intake in the rat. Wistar strain inbred rats from the Food and Drug Laboratories colony (Ottawa, Canada) were used in these experiments.

Experiment 1: The effect on the survival time of 40 weanling rats with an essential fatty acid deficiency which were fed D (as calciferol in propylene glycol) with and without a linoleate supplement is shown in Table 16. As can be seen in the table, none of the rats given D (10,000 IU/100 g BW and 40,000 IU/100 g BW) survived beyond two weeks. Supplemental linoleate increased the survival time only of those rats fed the larger dose of D.

Experiment 2: When 80 weanling rats fed a fat-free diet (see original paper for description) for two weeks (then divided into four groups) were then fed D with and without linoleate and linoleate alone (controls were given propylene glycol alone), it was found that the D alone depressed growth in the young rats. Large doses of D were not found to have any influence on the fatty acid composition of the liver.

Experiment 3: Young male rats fed the fat-free diet were divided into four groups of 20 rats each and given respectively 0.1 ml daily of four different fats (see Table 17). Ten rats from each group were fed 5000 IU D daily for three weeks. The results of this experiment are shown in Table 17. As can be seen, the D caused a decrease in weight gain and kidney calcification.

Table 14

Survival of Albino Rats Receiving Various Activated Sterols Daily Until Death. (3821)

Preparation	Experiment	No. of rats	Dose, mg/kg/day	Survival time, days: Range	Survival time, days: Avg. (median)	Equivalent toxic dose[a]
Vitamin D_2	A[b]	12	2.0	13-87	33 ± 5[c] (22)	3.60
Vitamin D_2	C	12	2.4	16-72	43 ± 3.3 (42)	
Vitamin D_2	D	10	3.0	11-82	33 ± 5.6 (25)	
Vitamin D_3	A	12	2.0	9-32	18 ± 1.5 (17)	2.30
Vitamin D_3	B	8	1.5	19-124	59 ± 10 (44)	
Vitamin D_3	B	8	2.0	16-43	29 ± 2.5 (28)	
Vitamin D_3	C	11	1.75	12-70	39 ± 4.3 (34)	
Vitamin D_3	D	10	2.25	7-74	27 ± 4.7 (20)	
Dihydrotachysterol	A	12	1.0	8-181[d]	34 ± 10 (18)	1.00
Dihydrotachysterol	C	12	1.12	9-67	24 ± 2.6 (20)	
Dihydrotachysterol	D	10	1.25	9-35	15 ± 5.4 (12)	

a = Mg/kg/day--permitting a median 20-day survival time, as estimated from the data of Experiments A, C, and D.
b = The experiment numbers are given in order to indicate which animals were run at the same time.
c = Probable error of the mean.
d = Animal sacrificed on the 181st day and serum Ca found to be 14.3 mg%. Judging from its condition the animal might still have survived for a considerable period of time.

Table 15

Final Serum Calcium Values of Albino Rats Medicated Daily with Various Activated Sterols for 14 Days. (3821)

Preparation	No. of rats	Dose, mg/kg/day	Serum calcium, mg% Range	Serum calcium, mg% Avg.	Dose required to produce serum Ca 15.3 mg%[a]
Vitamin D_2	8	2.0	13.1-17.3	15.74±0.38[b]	1.85
Vitamin D_3	8	1.0	13.5-15.4	14.61±0.23	1.2
Vitamin D_3	8	1.5	15.3-17.1	16.30±0.16	
Dihydrotachysterol	8	1.0	14.2-17.0	15.29±0.24	1.0

a = Estimated from the data, as mg/kg/day for 14 days, assuming that the rise above the normal level of 11.2 mg% is proportional to the log of the dose.
b = Probable error of the mean.

Table 16

Effect of Methyl Linoleate on the Survival Time of Rats Given Massive Doses of Vitamin D (4141)

Linoleate supplement	Vitamin D dose (IU/100 g body wt. per day) 10,000	40,000
	Survival time (days)	
None	8.0 ± 0.6*	6.6 ± 0.3
100 mg/day	10.0 ± 0.9	6.4 ± 0.3

* Mean ± S.E.

Table 17

Effect of Vitamin D and Various Fat Supplements on Weight Gain, Plasma Calcium, and Kidney Calcification (4141)

Supplements		Food cons. (g)	Wt. gain (g)	Plasma Ca (mg/100 ml)	Calcific.
Fat (0.1 ml/day)	Vitamin D (5000 IU/day)				
[illegible]	-	201 ± 9*	49 ± 4	8.78 ± 0.36	0.5
[illegible]	+	181 ± 6	31 ± 3	11.21 ± 0.45	2.5
[illegible]	-	213 ± 8	52 ± 4	9.37 ± 0.35	0.6
[illegible]	+	180 ± 9	31 ± 4	9.51 ± 0.32	4.0
[illegible]	-	221 ± 7	68 ± 4	8.17 ± 0.34	0.8
[illegible]	+	204 ± 6	47 ± 3	11.00 ± 0.46	3.1
[illegible]	-	221 ± 3	73 ± 2	9.76 ± 0.34	1.2
[illegible]	+	213 ± 8	62 ± 5	11.30 ± 0.36	3.6

* [illegible] ± S.E.

Experiment 4: In this experiment young male weanling rats were fed a fat-free diet supplemented daily with 0.1 ml butter fat, olive oil, safflower oil or menhaden oil. In each group ten of the rats were also given 5000 IU D per day. Again it was observed that D significantly decreased the growth of the rats (for details of the results see the original paper).

Experiment 5: The effect of various fat supplements on 100 linoleic-acid-depleted rats divided into five groups of 20 and given non-lethal doses of D (5000 IU per day to ten animals in each group) was measured. The results are shown in Table 18. All groups given D showed a weight loss and increased kidney calcification. The latter was not affected by the oil supplements.

9. In 1963, Constantinides (1126) reported producing true intimal foam cell lesions in rat arteries, particularly coronaries, within six weeks. The procedure used was the following:

a. Some lipemia was induced during the first two weeks by means of a cholesterol-thiouracil-cholate diet.

b. Viosterol was administered for three days during the third week.

c. Finally, three weeks more of the lipemia-producing diet was given.

The authors explanation was that by first adapting the rats to relatively harmless steroids their general resistance was increased to the much more toxic steroid, viosterol. This resulted in minimum arterial injury with maximal internal hyperplasia and lipophagia.

10. In 1965, Zemplenyi et al. (6354) fed locally bred male rats, 190-200 g, Hartroft's diet which induced lipid accumulations in aortic endothelium without connective tissue reactions. This later resulted in multiple thromboses and myocardial infarctions. They then repeated this experiment, adding 30,000 IU D_3 in oil daily for five or nine days (6353) and found additional connective tissue damage. The authors inferred that the connective tissue damage was a primary effect of the hypervitaminosis D_3 that would lead to secondary calcification.

11. In 1967, Creuss and Clark (1234) studied the effect of D on the phospholipids of the long bones of rats. A total of 33 male hooded rats (R.V.H. strain, ca. 110 g) were fed a laboratory diet; 18 animals received 15,000 IU D/100 g BW daily dissolved in sesame oil; the rest, the controls, were given an equal volume of sesame oil. Twelve experimental animals and 11 controls were sacrificed between days 14 and 18, while six experimentals and six controls were maintained for 24 days before sacrifice. It was found that:

Table 18

Effect of Vitamin D, Butter, Olive Oil, and Linoleate on Weight, Plasma Calcium, and Kidney Calcification in Essential Fatty Acid Deficient Rats (@141)

Supplements		Food cons. (g)	Wt. gain (g)	Plasma Ca (mg/100 ml)	Kidney Calcific.
Fat (0.1 ml)	Vitamin D (5000 IU/day)				
-	-	404 ± 13[a]	14 ± 5	9.23 ± 0.17	1.0
-	+	276 ± 12	-39 ± 4	11.61 ± 0.30	2.0
Butter	-	422 ± 11	33 ± 3	9.42 ± 0.21	0.5
Butter	+	277 ± 14	-27 ± 6	11.90 ± 0.20	2.2
Olive	-	412 ± 14	42 ± 5	9.20 ± 0.28	0.6
Olive	+	279 ± 15	-15 ± 5	11.68 ± 0.24	2.5
Olive and linoleate[b]	-	406 ± 12	42 ± 2	8.89 ± 0.27	0.3
Olive and linoleate[b]	+	289 ± 15	-10 ± 6	11.32 ± 0.29	3.0
Olive and linoleate[c]	-	428 ± 11	59 ± 4	9.06 ± 0.30	1.0
Olive and linoleate[c]	+	313 ± 17	-12 ± 5	10.94 ± 0.54	2.3

a = Mean ± S.E.
b = 25 mg methyl linoleate per 0.1 ml.
c = 50 mg methyl linoleate per 0.1 ml.

a. The D dosed rats did not gain weight as well as the controls.

b. The experimental rats showed a significant increase in the bone organic fraction.

c. The P^{32} uptake by the phospholipids in the treated animals was significantly increased, indicating an increased synthesis. (See results in Table 19 and 20.)

The authors suggested that the accumulation of lipid material may be related to the failure of the osteoid to calcify properly in hypervitaminosis D.

12. Fraser et al. (1896) produced kidney stones in rats with D. Seven female Wistar rats (between 140-190 g) were fed normal rat chow plus 10,000 IU (0.25 mg) of D_3 once a week by stomach tube for 45 days. The table below shows the numbers and types of stones produced.

Type of Stone	Numbers
$Mg\ NH_4\ PO_4 \cdot 6\ H_2O$	4
$Ca\ H\ PO_4 \cdot 2\ H_2O$	81
Calcium Oxalate $\cdot 2\ H_2O$ and $\cdot\ H_2O$	1
Amorphous calcium phosphate	1
Too small to examine	4
Total number	91

As can be seen from the table, the predominant stone type produced was calcium hydrogen phosphate. The average weight was about 73 mg. Further data from this report will be found in Biochemical Information, section V.

C. Rabbits

1. In 1957, Donath et al. (1536) studied the effect of various D preparations in rabbits. Five groups of rabbits respectively were given weekly intravenous injections of solutions of: a product of irradiated ergosterol, pure D_2 with vitamin A, or solvent alone.

It was observed that there was no significant difference in the effects of the different D preparations. Some of the effects were:

a. Slight falls in hemoglobin, and small but significant falls in red cell counts suggestive of incipient anemia.

b. Significant increases in serum cholesterol and alkaline phosphatase.

c. Significant increases in serum Ca, nonprotein nitrogen, urea and inorganic P.

Table 19

Uptake by Bones of Hypervitaminotic D Animals
14-18 Days on Treatment (1234)

	Control (11 animals)	Hypervitaminosis D (12 animals)
Final wt. (g)	170 ± 4.2	116 ± 6.3***
Bone - Ash (% dry, fat-free wt.)	65.3 ± 1.47	58.5 ± 1.34***
Bone - Organic fraction (% dry, fat-free wt.)	34.7 ± 1.45	41.5 ± 1.34***
Phospholipid - Bone (mg/g)	1.97 ± .22	3.24 ± .37***
Phospholipid - Organic fraction (mg/g)	5.64 ± .35	7.93 ± .58**
P^{32} uptake - Counts/min/g bone	7629 ± 1502	15,701 ± 2041**
P^{32} uptake - Counts/min/mg phospholipid	3441 ± 764	6418 ± 1460**

*** Significance at .1% level (p<.001).
** Significance at 1% level (p<.01).
* Significance at 5% level (p<.05).

± Standard error.

Table 20

Phospholipids of Bones by Hypervitaminotic D
Animals 24 Days on Treatment. (1234)

	Control (6 animals)	Hypervitaminosis D (6 animals)
Final wt.	182 ± 5.2	124 ± 5.8***
Bone - Ash (% dry, fat-free wt.)	65.0 ± 1.37	58.4 ± 1.46***
Bone - Organic fraction (% dry, fat-free wt.)	35.0 ± 1.35	41.6 ± 1.47***

	mg/g organic fraction	% of total phospholipid	mg/g organic fraction	% of total phospholipid
Total phospholipid	7.03 ± .42		11.60 ± .64***	
Lecithin	3.63 ± .24	51.6	5.99 ± .28***	51.1
Phosphatidylethanolamine	2.22 ± .18	31.5	3.01 ± .20**	26.5
Sphingomyelin	.53 ± .09	7.6	1.19 ± .11**	10.3
Lysolecithin	.30 ± .07	4.3	.60 ± .09	5.1
"X"	.16 ± .04	2.2	.46 ± .06	4.1

*** Significance at .1% level (p<.001).
** Significance at 1% level (p<.01).
* Significance at 5% level (p<.05).

± Standard error.

d. Considerable weight loss particularly in those animals given vitamin A.

e. The mean Ca contents of the aorta and kidney were 6.9 and 2.7 %, as compared to less than 0.04 % in controls.

f. Ca contents of the femur and skull in the experimental animals were low.

g. Rabbits which died had uremic symptoms.

j. In 1958, Hass et al. (499) studied histologically the distribution and evolution of lesions of the vascular system in rabbits given excessive doses of irradiated ergosterol. Male albino rabbits (3 months old, about 5 pounds) were dosed with varying amounts of viosterol. There were 54 experimental animals and 75 controls used. The dosages studied were:

a. 300,000 units given in three equal doses at two day intervals for eight days. This was the minimum dosage for production of significant general calcinosis.

b. 500,000 to 600,000 units administered in periodic equal doses for at least three weeks. This dose produced generalized calcinosis of increasing severity.

c. Bi-weekly administration of 100,000 units continued beyond three weeks. This regime generally led to anorexia, loss of weight and death within about six weeks.

The main observations were:

a. Bones - In general they were more brittle than normal and varied with the severity and duration of the hypervitaminosis D.

b. Calcium deposits - The amount and distribution of abnormal calcium deposits in the soft tissues also varied with the duration and severity of the hypervitaminosis D. Calcium was most conspicuously distributed in the aorta and its main branches, somewhat less so in the kidney. Muscle and the respiratory tract were other common locations of calcium deposits. In the latter, tracheal and bronchial cartilages were frequently rigid and calcified. Also calcium was found in the walls of the pulmonary veins. Gross calcium traces were also noted in other organs.

c. The pathological processes attributable to hypervitaminosis D varied with viosterol dosage, length of period between doses, duration of the

regime and the occurrence of intercurrent infections. (For details of the microscopic pathological changes observed, see the original paper.)

The authors concluded that:

a. Administration of D in amounts just sufficient to produce pathologic changes led to mineralization of certain tissues which do not normally calcify.

b. With somewhat larger doses, abnormal mineralization of tissues was often preceded and accompanied by structural changes of a degenerative type.

c. Still larger doses excited early inflammatory reactions in cardiac and skeletal muscle only.

d. Studies of restorative phases of the disorder gave evidence of reversibility and resistance to progression of these processes.

3. In 1963, Matsuda and Kato (3770) compared the action of D_2 and D_3 on the calcification of dentin in the rabbit. Pure preparations of D_2 and D_3 were given s.c. to normal rabbits in various doses with the following results:

Vitamin D_2

a. Each of three overdoses, 400,000 IU/kg, 100,000 IU/kg and 10,000 IU/kg, were given s.c. once daily for three consecutive days to five rabbits.

The animals died five to seven days after injection following anorexia and weight loss. Only the highest dosage slightly inhibited tooth calcification. The other two dosages accelerated calcification. Serum calcium decreased at all dosage levels and serum inorganic phosphate increased. Serum protein decreased appreciably.

b. Eight rabbits were given 5,000 IU/kg s.c. daily for three consecutive days. Calcification was accelerated without side effects.

No change was noted in the degree of dentin formation. Serum calcium was inconsistent: serum inorganic phosphate gradually decreased, and serum protein decreased.

c. Five animals were given 3,000 IU/kg s.c. once daily for three consecutive days.

The dose was only 40% effective in promoting calcification.

Vitamin D_3 a. Ten animals were injected s.c. once daily for three consecutive days with 800,000 IU/kg.

No toxic effects were observed. Serum calcium increased gradually as well as serum protein. Calcification as indicated by formation of a deep-blue stained layer increased as the ratio between calcium and phosphate decreased below normal and alkaline phosphatase activity diminished. (This was also the case with D_2.)

b. Five animals were injected s.c. with 400,000 IU/kg once daily for three consecutive days.

This dose was 40% effective.

The authors concluded that:

a. The range for an effective dose of D_2 without toxicity was 3,000 to 10,000 IU/kg.

b. An overdose of D_2 s.c. had an accelerating effect on calcification except when the dose was 400,000 IU/kg when it was inhibitory.

c. D_2 was superior to D_3 in promoting dentin calcification in normal rabbits.

d. The range of effective doses of D_3 was wider than of D_2.

e. With both D vitamins hypercalcification was accompanied by decreased alkaline phosphatase activity. Table 21 compares the effects of both these vitamins on calcification, blood and urinary components.

4. In order to determine whether the electrocardiographic changes in patients with infantile hypercalcemia resulted from myocardial lesions, in 1965 Coleman (1083) administered large doses of D to rabbits.

Vitamin D (40-420 IU D_2 and 100 to 770 IU D_3/g BW) in arachis oil was administered orally for 10 days to 18 rabbits (nine D_2, nine D_3, nine control) and was completed by six weeks of age. Seven of the animals died: two in congestive heart failure (D_2 347 units/g: D_3 103 units/g) showing hypertrophy of muscle fibers and severe aortic lesions: four others had focal myocardial lesions (D_2 230 units/g, 40 units/g; D_3 770 units/g, 660 units/g). One rabbit which became ill and was killed was the only one with coronary artery lesions.

An aortic lesion was present in every rabbit given vitamin D but none in the controls. In ten of these rabbits (D_2 40-420 units/g; D_3 100-770 units/g)

Table 21

Comparative Studies on the Effects of Vitamins D_2 and D_3 upon Calcification of Dentin, and Urinary and Blood Components of Normal Rabbits. (3770)

	Calcification accelerating dose	Serum calcium	Serum phosphate	Serum alkaline phosphatase	Serum protein level	Blood pH	Daily output of urinary calcium	Daily output of urinary phosphate
D_2	5,000 IU/kg subcut. 3 time	No definite tendency	Increase	Decrease	Decrease	Little change	Increase	Increase
D_3	800,000 IU/kg subcut. 3 time	Gradual increase	Increase	Decrease	Increase	Little change	Increase	Transient decrease

there were lesions immediately subjacent to the mural endocardium of the left ventricle, left atrium or right ventricle. Serum calcium levels in excess of controls were found as long as 32-38 weeks after the cessation of the D administration.

The author concluded that the possibility exists that congenital endocardial fibroelastosis and the myocardial lesion of fibrocystic disease of the pancreas are related to vitamin D.

5. In 1966, Friedman and Roberts (1947) explored the relationship between hypervitaminosis D in the mother rabbit and the development of supravalvular aortic stenosis in the offspring. (Here only the production of hypervitaminosis in the mother is abstracted. The transplacental effects are discussed on pp. 154-157.)

The experimental procedure was:

a. Adult white New Zealand rabbits were mated, with each female having a different mate.
b. Starting with the day after copulation, eight females on a stock diet were given D i.m. (activated ergosterol in cottonseed oil) on alternate days for a total of 1.5 million units.
c. Three groups of five females on the stock diet, each were given D i.m. for 30 days after copulation, for total amounts of 2.5, 3.5 and 4.5 million units respectively.
d. Controls were eight females on the stock diet.
e. Three additional females were made D deficient by feeding them a stock diet without D supplements.

The observations of the mothers showed the following:

a. The D level in the mothers given 1.5 million units was 7 times greater than that of the controls.
b. There were no statistically significant differences in measurements of serum calcium levels and phosphorus values between mothers and controls.
c. All of the females given 2.5, 3.5 and 4.5 million units of D died within 65 days after their first vitamin injection.
d. All of the above excessively dosed females which conceived either aborted during the first 12 days of pregnancy or delivered macerated fetuses.

e. The entire aorta of each of the 2.5, 3.5 and 4.5 million units dosed rabbits, showed advanced changes including irregular depressions in the internal wall, focal calcium deposits, foci of degeneration and necrosis of the aortic media.

f. The aortae of the mothers given the smaller 1.5 million unit dose, showed similar but less striking changes, most pronounced in the proximal portion.

D. Chicks

In 1934, Waddell (6048) investigated the comparative effectiveness of irradiated cholesterol and ergosterol in preventing leg weakness in chicks. A number of experiments were carried out as follows:

Irradiated crude cholesterol: In the first experiment eight weeks in duration, the material, suspended in corn oil, was added to the basal diet of chicks (specifications not given) in proportions so that each group received 20, 80 and 320 rat units per 100 g of diet respectively (one rat unit was found to be approximately 0.75 mg). Another group of chicks was given 10 rat units of cod liver oil per 100 g of diet and four more groups received 10, 40, 150 and 640 rat units of irradiated ergosterol per 100 g diet.

The results of this experiment showed that irradiated cholesterol at all levels protected against leg weakness. Growth and outward appearance compared favorably to the cod liver oil fed group. While at the lower levels irradiated ergosterol proved relatively ineffective. Only 640 rat units produced a normal appearing group. (A second more extensive repeat of the above experiment is summarized in Table 22.)

The author concluded from these experiments that irradiated cholesterol is as potent as the vitamin D of cod liver oil in preventing leg weakness in chicks.

Irradiated pure cholesterol: 0.5 mg contained one rat unit. White leghorn chicks (no details given) were fed the equivalent of 20, 40 and 60 rat units per 100 g of diet. This was compared as before with other supplements, including crude cholesterol.

The results showed excellent growth and calcification resulting from the irradiated pure cholesterol.

Fractionation experiments: Experiments were carried out in which the active fraction was separated out and the recovered material irradiated. This procedure

Table 22

Summary Showing Difference in Efficacy of Irradiated Crude Cholesterol and of Irradiated Ergosterol Alone and in Presence of Non-Irradiated Crude Cholesterol (6048)

Group No.	Supplement to basal Ration I*	Rat units per 100 g ration	Average weight at 8 weeks g	Bone ash at 8 weeks** per cent	Remarks
1	None		285	35.2	All symptoms of leg weakness evident; all squat
2	0.25% cod liver oil	10	626	43.7	Chicks healthy; very active
3	10 mg irradiated crude cholesterol	20	633	44.6	Chicks healthy; very active
4	30 mg irradiated crude cholesterol	60	600	43.0	Chicks healthy; condition excellent
5	90 mg irradiated crude cholesterol	180	614	43.5	Chicks healthy; condition excellent
6	Irradiated ergosterol +10 mg crude cholesterol	10	364	38.5	More than half of chicks squat
7	Irradiated ergosterol +30 mg crude cholesterol	30	311	35.4	About 3/4 of group squat; condition poor
8	Irradiated ergosterol +90 mg crude cholesterol	90	350	36.7	About 3/4 of group squat; condition poor
9	Irradiated ergosterol	90	362	36.9	All but 3 squat; condition poor

* All chicks were maintained on the basal diet for 1 week before additions, as noted, were made.
** Figures for percentage of bone ash were obtained on ten chicks from each group.

was followed several times (see original paper for details) and the resulting fractions tested. These experiments are summarized in Tables 23 and 24.

The author concluded that the repeated irradiation of cholesterol (as described in the paper) did not produce any appreciable amounts of antirachitic substances for chicks nor any substance which had a supplementing effect with irradiated ergosterol. Also that the high potency of the irradiated cholesterol was the property of the activated "pro-vitamin" of cholesterol.

Various mixtures: Mixtures of cholesterol and ergosterol were used in another series of experiments. These are summarized in Tables 24 (groups 10, 11 and 12) 25 and 26.

The author's conclusions were that pure ergosterol heated and irradiated in the presence of cholesterol did not produce any more effective vitamin than that from irradiated ergosterol alone. Also that ergosterol behaved differently from the "pro-vitamin" in cholesterol. The author then concluded that the "pro-vitamin" constituent of cholesterol differed from ergosterol. This conclusion did not agree with the belief widely held in the 1930's, when this paper was written, that ergosterol was the main precursor of D activity in the body.

E. Dogs

1. Taylor and Weld (5713) investigated the apparently greater susceptibility of growing animals to irradiated ergosterol. Two groups of pups were used. The first group of five animals (ca. 4 mos. old, 2.71 to 4.76 kilos) was orally administered ergosterol which was diluted 1:10 with corn oil. The potency of the diluted material was ten times that of its therapeutic potency as originally assayed by the manufacturers. The dose schedule and effects of the dosage are summarized in Table 27.

The authors concluded (see Table 27) that ergosterol predisposed the young dog to intussusception. They subsequently observed a large proportion of dogs dying from this cause following ergosterol overdosage. They further concluded that in young dogs irradiated ergosterol induced a disturbance in the motor mechanisms of the bowel which predisposed to the condition, and that intussusception was an effect of the vitamin D administration which usually supervened early and caused death before the other signs of overdosage were apparent.

The second group of six animals (2-2 1/2 mos. old) was given similar doses of undiluted irradiated ergosterol. All these animals died within two weeks.

Table 23

Summary Showing Difference in Efficacy of First, Second and Third Filtrate Fractions from Repeated Irradiation of Pure Cholesterol (6048)

Group No.	Supplement to basal Ration I*	Rat units per 100 g ration	Average weight at 8 weeks	Bone ash at 8 weeks	Remarks
			g	per cent	
1	First filtrate fraction (= 5 mg)	10	641	45.0	Chicks healthy
2	Same (= 10 mg)	20	661	44.6	Chicks healthy
3	Same (= 15 mg)	30	719	44.6	Chicks healthy
4	Second filtrate fraction (= 5 mg)	<1	582	40.2	Unsteady and staggering
5	Same (= 10 mg)	<2	544	38.2	Unsteady and staggering
6	Same (= 15 mg)	<2	619	43.0	Some unsteady
7	Third filtrate fraction (= 5 mg)	<1	536	38.1	Marked leg weakness
8	Same (= 10 mg)	<1	606	40.4	Leg weakness evident
9	Same (= 15 mg)	<1	588	40.3	6 unsteady

* When this experiment was started our supply of chicks was limited; hence, no group was included which received the basal diet alone. All chicks were maintained on the basal ration 10 days before additions were made.

Table 24

Summary Comparing Potency of First and Fifth Filtrate Fractions and of an Irradiated Mixture of Cholesterol and Ergosterol (6048)

Group No.	Supplement to basal Ration I*	Rat units per 100 g ration	Average weight at 8 weeks	Bone ash at 8 weeks	Remarks
			gm	per cent	
1	None		351	34.0	Almost all squat
2	0.25 per cent cod liver oil	10	712	44.2	Condition very good
3	First filtrate fraction (= 5 mg)	10	614	43.3	Good condition
4	Same (= 10 mg)	20	659	44.4	Very good condition
5	Same (= 15 mg)	30	626	44.3	Very good condition
6	Fifth filtrate fraction (= 15 mg)	<1	418	37.7	More than half squat
7	Same (= 50 mg)	<1	448	36.6	9 squat
8	Fifth filtrate fraction (= 15 mg) + irradiated ergosterol	30	479	38.6	9 squat
9	Fifth filtrate fraction (= 15 mg) + irradiated ergosterol	30	479	38.6	6 squat
10	Irradiated mixture of cholesterol and ergosterol (95:5)	10	432	36.4	8 squat
11	Same	30	422	37.0	8 squat
12	Same	90	536	36.8	10 squat

* All chicks were maintained on the basal ration for 2 weeks before supplements were added.

Table 25

Summary Comparing Efficacy of Various Supplements Which Included Irradiated Mixture of Cholesterol and Ergosterol (6048)

Group No.	Diet and supplement*	Rat units per 100 g ration	Average weight at 8 weeks	Bone ash at 8 weeks	Remarks
			g	per cent	
1	Basal Ration I	0	350	36.1	Almost all squat
2	Same + 0.25 per cent cod liver oil	10	642	43.0	Excellent
3	Same + irradiated ergosterol	30	353	36.1	12 squat
4	Same as for Group 3	90	485	39.7	8 squat
5	Same + filtrate from irradiated crude cholesterol	10	655	45.9	Excellent
6	Same as for Group 5	20	617	43.1	Condition very good
7	Same as for Group 5	30	755	44.7	Excellent
8	Same + irradiated pure cholesterol	10	612	43.7	Condition very good
9	Same as for Group 8	20	565	43.6	Condition very good
10	Same as for Group 8	30	614	42.6	Excellent
11	Same + filtrate of irradiated cholesterol + 0.1 per cent ergosterol	10	510	40.4	4 squat
12	Same as for Group 11	20	421	37.6	10 squat
14	Basal Ration II	0	227	30.7 (D.) (6)** 33.6 (L.) (8)	All squat
15	Same + 0.5 per cent cod liver oil	20	534	40.5	Condition good
16	Same + filtrate from irradiated crude cholesterol	20	618	42.5	Condition very good
17	Same + irradiated pure cholesterol	20	566	41.6	Condition good

* All chicks in this experiment were maintained on basal Ration I for 2 weeks, additions then being made as noted. Groups 14 to 17 were changed to basal Ration II at the same time.

** Bones were removed from six chicks which died (D.) during the last week of the experiment and were kept in a separate group. The eight remaining (L.) at the end of the experiment were grouped and the ash was determined separately.

Table 26

Summary Comparing Efficacy of Irradiated Ergosterol, Two Heated and Irradiated Mixtures of Cholesterol and Ergosterol, and a Fraction of Irradiated Crude Cholesterol (6048)

Group No.	Supplement to basal Ration II	Rat units per 100 g ration	Average weight at 6 weeks	No. of chicks		Bone ash at 6 weeks*	Remarks
				At beginning	At End		
			g			per cent	
1	None	0	135	16	4	28.6 (13)	All survivors squat
2	0.25% cod liver oil	10	428	16	15	41.6	Slight unsteadiness in few
3	0.5% cod liver oil	20	410	15	15	41.4	Healthy
4	Irradiated ergosterol	30	164	15	10	30.4 (15)	All except 3 squat
5	Same	90	209	15	13	31.6 (14)	About 3/4 of group squat
6	Same	270	396	15	13	37.2 (14)	4 unsteady, 1 squat
7	Filtrate irradiated, heated (2 hrs.) mixture cholesterol + ergosterol	10	140	14	5	28.7 (9)	All survivors squat
8	Same	30	175	15	4	27.1 (13)	All survivors squat
9	Same	90	195	15	14	30.5	All but 3 squat
10	Same	270	389	15	15	35.6	4 or 5 unsteady
11	Filtrate irradiated, heated (4 hrs.) mixture cholesterol + ergosterol	10	134	14	5	28.9 (12)	All survivors squat
12	Same	30	194	15	10	30.3 (15)	Almost all squat
13	Same	90	239	15	14	33.1 (15)	About half squat
14	Same	270	432	15	15	39.6	Slight unsteadiness in few
18	Filtrate from irradiated crude cholesterol	2	175	15	13	30.9 (15)	All squat
19	Same	5	361	15	15	40.2	Slight unsteadiness in few
20	Same	10	391	15	15	41.1	Slight unsteadiness in few

* Since most of the fatalities occurred during the last 10 days of the experiment, bones were removed from those chicks that died in that time and included with the bones from the survivors in determining percentage bone ash. Figures in parentheses indicate number of bones used. Where there are no parentheses surviving chicks contributed all bones.

Table 27

Effect of Irradiated Ergosterol on Pups Above 4 Months Old (5713)

No. of animal	Weight before administration in kilos	Daily dose of irradiated ergosterol 10,000 X in c.c. Total	Per kilo	Equivalent of 250 D per kilo	X T* per kilo	Number of days from commencement of administration to death	Serum calcium mgm. per 100 c.c. and symptoms	Weight at, or shortly before, death in kilos	Post-mortem findings
1	2.71	0.3	0.10	10	83	11	Hypercalcaemia (18 mgm.) conjunctivitis, vomiting, diarrhoea, blood in stools.	1.7	Flushing of gastric mucosa with small extravasations of blood. Haemorrhagic areas in lungs.
2	3.77	0.25	0.06	6.6	55	36	Hypercalcaemia (16 mgm.) conjunctivitis and inflammation of lids with loss of eyelashes. Profound emaciation. Vomit- and diarrhoea in early part of experiment.	2.77	Stomach and intestines apparently normal.
3	4.76	0.20	0.04	4.0	33	59	Hypercalcaemia (14 mgm.) shortly after commencement followed by recovery to normal.	2.31	Stomach and intestines apparently normal.
4	3.69	0.15	0.04	4.0	33	15	Calcium 11 mgm. Loss of weight. No other definite symptoms until 24 hours before death, when vomiting and great weakness, ending in collapse occurred.	2.57	Intussusception of lower 12 in. of ileum into colon, invaginated bowel, haemorrhagic and sections gangrenous.
5	4.11	0.1	0.02	2.0	17	60	Slight rise in serum calcium during first 2 weeks. Fall after this which reached normal 2 weeks later.	2.72	Nothing abnormal noted in viscera.

*T = maximal therapeutic dose (0.12 c.c. 250 D per kilo).

The dose schedule and effects of the dosage are summarized in Table 28.

A second series of experiments was carried out because the authors considered the possibility that the stock diet fed the dogs might have been inadequate and might have needed vitamin fortification to enable the animals to better resist the effects of ergosterol overdosage. In this experiment six police pups from the same litter, about two months old, were fed the vitamin fortified diet and then dosed with irradiated ergosterol. The dose schedule and effects on weight and survival are summarized in Table 29. As can be seen, at the end of three weeks most of the animals lost weight. The authors noted that the animal receiving the smallest dose showed a steady decline in weight after the first five weeks until death at two months. This animal's dose was only four times the maximal therapeutic dose. The authors stated that even though caution should be observed when extrapolating data from animals to man, these results suggested that the administration of irradiated ergosterol to infants in amounts not greatly exceeding those recommended at the time of writing (1952) might be dangerous.

A further conclusion drawn by the authors was that hypercalcemia did not always occur when animals received comparatively small, though still toxic, doses of irradiated ergosterol over long periods. Therefore, they felt that other less reliable criteria of overdosage such as loss of body weight, lowered vitality and shorter survival time were useful and valid. They considered that when the total number of animals employed was taken into account, the deterioration of the animals invariably following ergosterol administration left little room for doubt that overdosage was the cause of death.

In a final series of experiments eight dogs, somewhat older (3 months) than those in the previous groups, were treated with irradiated ergosterol. The dose schedule and survival time are summarized in Table 30. In this group there was not a complete correspondence between dose size and survival time. The authors pointed out that they have found wide variation in resistance to overdosage among animals, particularly older animals. For example, in some animals a daily dose less than ten times the maximal infant dose proved toxic, while one animal was unaffected by a daily dose 25 times this maximal dose. Controls (numbers not given) were used in all the above experiments.

2. In 1937, Stack et al.(5512) studied the effect of massive doses of D on dogs. In this experiment with 64 healthy adult dogs, D in the form of a

Table 28

Effect of Irradiated Ergosterol on Pups 2-2 1/2 Months Old. (S713)

No. of animals	Weight before administration in kilos	Daily dose of irradiated ergosterol 10,000 X in c.c. Total	Per kilo	Equivalent of 250 D per kilo	X T* per kilo	Number of days from commencement of administration to death	Serum calcium mgm per 100 c.c. and symptoms	Weight at death in kilos	Post-mortem findings
100	1.18	0.35	0.29	29.0	240	8	Hypercalcaemia (20.3 mgm.), vomiting, great weakness, especially of hind quarters, blood highly concentrated.	0.90	Deep flushing of gastric mucosa haemorrhagic patches in lungs, intussusception of 3 in. of lower ileum into colon, bone marrow haemorrhagic.
101	1.25	0.30	0.24	24.0	200	11	Hypercalcaemia (22.6 mgm.), emaciation, weakness leading to complete prostration.	0.97	Ileum contracted, a thin cord, caecum widely dilated, conditions appear preparatory for development of intussusception. Lungs show haemorrhagic areas 1 cm. square, bone marrow haemorrhagic.
102	1.25	0.25	0.2	20.0	166	12	Hypercalcaemia probably present, but calcium determinations not made. Symptoms similar to preceding.	0.99	Intussusception of 3 in. of lower ileum. Gastric mucosa shows flushing only.
103	1.32	0.20	0.14	14.0	116	14	Hypercalcaemia (24.0 mgm.). Symptoms similar to those of preceding animals	0.90	Gastric mucosa injected, a few ecchymotic areas. Bowel filled with dark brown fluid. Lungs show well-defined haemorrhagic areas. A section of ileum about 3 in. long deeply congested and showing a haemorrhagic area 1/2 in. long. The mesentery of this section of the ileum injected, vessels dilated. Ileum on either side of this area is quite pale and normal in appearance.
104	0.91	0.15	0.16	16.0	136	6	Diarrhoea, weakness, particularly of hindquarters, comatose last few hours of life.	0.78	Lungs haemorrhagic, stomach mucosa colour normal, bone marrow haemorrhagic.
105	1.56	0.10	0.06	6.0	50	13	Hypercalcaemia (19.4 mgm).	1.03	No haemorrhages in lungs. Stomach bright red in pyloric region, duodenum pale, bone marrow haemorrhagic, ileum firmly contracted, in sections separated by dilated pouches, but there is no intussusception.

*T = maximal therapeutic dose

Table 29

Effect of Irradiated Ergosterol on Pups About 8 Weeks Old, Fed on a Diet Containing Adequate Vitamins B and C (9713)

Puppy No.	Weight in Kilos. Jan. 26, 1931	Feb. 5	Feb. 14	Mar. 2	Mar. 9	Mar. 16	Mar. 23	Mar. 30	Daily dose per kilo 10,000 X in c.c. from	Daily dose in equivalent of 250 D in c.c.	X T*
1	2.39	2.20	Died Feb. 13 Wt. 1.60	---	---	---	---	---	Jan. 26, 1931 0.05	5	42
2	2.53	2.38	2.33	2.5	Died Mar. 9 Wt. 1.75	---	---	---	0.04	4	33
3	2.24	2.19	Died Feb. 14 Wt. 1.62	---	---	---	---	---	0.03	3	25
4	2.32	2.25	2.30	2.61	2.52	Died Mar. 14 Wt. 1.68	---	---	0.02	2	17
5	2.40	2.45	2.21	1.89	Died Mar. 3 Wt. 1.6	---	---	---	0.01	1	8
6	2.40	2.46	2.24	2.49	2.30	2.25	2.04	Died Mar. 27 Wt. 1.32	0.005	0.5	4

*T = maximal therapeutic dose

Table 30

Effect of Irradiated Ergosterol on Pups about 3 Months Old, Fed on a Diet Containing Adequate Vitamins B and C. (5713)

Pup No.	Weight in kilos	Survival time, weeks	Dose 10,000 X per kilo in c.c.	Equivalent in 250 D per kilo in c.c.	X T.*
II	2·29	10	0·07	7·0	58
III	2·27	4	0·06	6·0	50
VIII	1·20	6	0·04	4·0	35
X	1·60	Until conclusion of experiment	0·03	3·0	25

* T = maximal therapeutic dose.

solution of activated ergosterol in corn oil (1,000,000 units per g) or calciferol dissolved in corn oil was administered largely per os (a few animals, number not stated, received intravenous injections) in daily doses ranging from 15,000 to 500,000 units/kg BW. An effort was made to adjust the dose to decreasing body weight in order to keep the ratio between the dose and the weight of metabolizing tissue fairly constant.

The experiment is summarized in Table 31. The figures in the second column represent the number of days the animals survived the treatment. Those animals which did not die were sacrificed within three days following the last dose. The average survival times were:

a. With amounts greater than 50,000 units daily, 12 days.

b. With amounts between 20,000 and 50,000 units, 39 days.

c. With 20,000 units or less, 68 days.

The mean Ca content of the kidneys of normal dogs is 85 mg/100 g dried tissue. The average content of calcium in the kidneys of the D fed dogs was found to be as follows:

a. With a daily dose greater than 50,000 units/kg BW, 564 mg/100 g dried tissue.

b. With a daily dose between 20,000 and 50,000 units/kg BW, 921 mg/100 g dried tissue.

c. And with a daily dose of 20,000 units/kg BW or less, 183 mg.

The authors speculated that the lower average in the first group may have been related to the shorter survival time.

Table 31

Observations on Dogs Receiving Vitamin D (5512)

No.	1000 units/ K/day	Days	Kidney Mg Ca/ 100 gm Dry Tissue	Max. Blood Ca	Microscopic		Wt. Loss Per Cent	Other Symptoms of Toxicity	
					Cell. Degen.	Ca Stain			
1	500	8	671	19.90	5	5	40	Severe	Died in coma
2	500	9	212	21.60	5	5	32	Severe	Died in coma
3	500	9	---	---	-	-	28	Severe	Died in coma
4	500	11	---	---	-	-	38	Severe	Died in coma
5	200	18	---	---	-	-	44	Severe	Died in coma
6	200	10	---	---	-	-	34	Severe	Died in coma
7	130	7	598	16.36	4	3	30	Severe	Died in coma
8	125	12	52	23.30	1	0	23	Mild	Died of distemper
9	100	30	110	16.30	0	0	7	Mild	Died of distemper
10	100	6	676	14.98	3	1	19	Severe	Died of distemper
11	100	20	---	---	-	-	23	Severe	Died in coma
12	100	13	---	---	-	-	17	Moderate	Found dead
13	60	7	340	23.36	?	0	24	Severe	Died of distemper
14	60	13	540	23.29	1	1	20	Severe	Died in coma
15	60	8	685	18.16	-	-	9	Severe	Found dead
16	60	7	800	19.56	4	2	+17	Mild	Found dead
17	60	12	865	24.50	-	-	0	Severe	Found dead
18	60	13	1221	31.06	5	5	37	Severe	Died in coma
19	60	20	---	---	-	-	42	Severe	Died in coma
20	60	10	---	---	-	-	30	Severe	Died in coma
21	50	17	3464	27.00	5+	5+	15	Severe	Died in coma
22	50	43	119	18.90	1	0	21	Moderate	Found dead
23	50	35	---	---	-	-	10	Moderate	Allowed to recover
24	50	12	---	---	-	-	28	Severe	Died in coma
25	50	24	---	---	-	-	19	Severe	Died in coma
26	48	35	47	21.50	0	0	6	Mild	Fair condition when killed
27	38	10	2200	16.02	5	5	18	Severe	Died in coma
28	37	47	115	23.16	?	0	48	Mild	Good condition when killed except emaciated
29	35	73	1148	19.30	5	5	60	Severe	Died in coma
30	35	33	693	15.60	5	5	--	Severe	Poor condition when killed
31	35	23	597	16.47	5	5	35	Severe	Poor condition when killed
32	35	8	407	22.74	1	?	17	Mild	Died of distemper
33	35	60	---	---	-	-	26	Mild	Fair condition when killed
34	35	54	---	---	-	-	40	Moderate	Found dead
35	35	30	---	---	-	-	29	Severe	Found dead
36	35	26	---	---	-	-	42	Severe	Found dead

Table 31 (cont.)

No.	1000 units/ K/day	Days	Kidney Mg Ca/ 100 gm Dry Tissue	Max. Blood Ca	Microscopic		Wt. Loss Per Cent	Other Symptoms of Toxicity	
					Cell. Degen.	Ca Stain			
37	25	33	1214	19.38	4	4	13	Moderate	Good condition when killed
38	25	62	131	15.80	1	1	15	Mild	Good condition when killed
39	25	70	---	---	-	-	18	?	Good condition when killed
40	25	79	---	---	-	-	8	Moderate	Found dead
41	25	50	---	---	-	-	22	Severe	Died in coma
42	25	56	---	---	-	-	5	Severe	Died in coma
43	25	16	---	---	-	-	20	Severe	Died of distemper
44	20	38	228	13.26	0	0	0	0	Good condition when killed
45	20	41	186	12.90	0	0	3	0	Good condition when killed
46	20	67	174	11.78	0	0	5	Mild	Good condition when killed
47	20	83	203	12.02	0	0	0	0	Good condition when killed
48	20	120	93	11.00	0	0	+7	0	Good condition when killed
49	20	60	---	---	-	-	0	Mild	Died of distemper
50	20	93	---	---	-	-	0	0	Good condition when killed
51	20	80	---	---	-	-	7	?	Allowed to recover
52	20	55	---	---	-	-	0	0	Good condition when killed
53	20	40	---	---	-	-	0	0	Good condition when killed
54	15	62	212	16.85	?	0	5	Slight	Died of distemper
55	15	136	147	11.83	0	0	0	Slight	Good condition when killed
56	15	70	86	12.15	0	0	0	0	Good condition when killed
57	15	153	262	10.80	0	0	+16	0	Good condition when killed
58	15	56	248	11.50	0	0	0	0	Good condition when killed
59	15	61	---	---	-	-	+12	0	Good condition when killed
60	15	61	---	---	-	-	0	0	Good condition when killed
61	15	67	---	---	-	-	0	0	Good condition when killed
62	15	90	---	---	-	-	+10	0	Good condition when killed
63	15	47	---	---	-	-	0	0	Good condition when killed
64	15	30	---	---	-	-	5	0	Good condition when killed

Other observations made were:

a. In 36 out of 43 dogs receiving more than 20,000 units per kg per day, loss of weight was marked.

b. In only four of the 64 dogs examined was there any indication of medial thickening in the arteries.

c. With eight exceptions, all of the 43 dogs receiving more than 20,000 units/kg/day died spontaneously.

The authors concluded that:

a. Up to 20,000 units/kg/day of D administered daily for periods up to 153 days was not seriously injurious to dogs.

b. Since crystalline calciferol in corn oil (40,000,000 units per g) was as toxic as activated ergosterol, the toxic effects seen could be characterized as true hypervitaminosis D.

In a second experiment, 18 dogs were brought to an extremely toxic stage by administration of D (see Table 32 for amounts given) and then the administration was stopped. Some of the toxic effects were weight loss, anorexia, listlessness, paralysis, and prostration. Eight of the dogs, not shown in the table, died within two to seven days of the termination of the treatment. The dogs which didn't die showed evidence of recovery after various periods. These were then sacrificed and examined. The results are summarized in Table 32.

The authors conclusions from these experiments were:

a. The dogs could recover from extreme stages of D toxicity, and injured tissue could be repaired.

b. The degree of toxicity did not seem to be dependent on the total amount of D administered or on the size of the daily dose.

c. Sensitivity appeared to be lessened during the winter months.

d. Diet was an important factor in conditioning toxicity.

e. The concentration of plasma Ca was not closely related to toxicity.

3. In 1947, Hendricks et al.(2547) observed the effects of excess D on young dogs fed diets similar in Ca content to the diets of infants. The experiment which used 13 purebred, five to eight-week old cocker-spaniels, extended over 10 months.

The D sources used were: irradiated ergosterol (300,000 IU per g) halibut liver oil (1,430 IU/g and 160,000 IU/g in two lots); tuna liver oil (61,000 IU/g); and delsterol (300,000 IU/g). These were administered daily by

Table 32

Observations on Dogs Brought to Extreme Toxicity with D (5510)

No.	Days	Recovery Days	Units/ K/Day	Kidney Ca	Max. Blood Ca	Microscopy		Wt. Loss Per Cent	
						Cell. Degen.	Ca Stain		
1	24	126	15,000	257	18.59	0	0	6	Good condition
2	15	9	20,000	48	19.89	0	0	13	Good condition
3	80	102	20,000	212	17.41	0	0	7	Good condition
4	10	48	25,000	323	22.13	0	0	30	Good condition
5	5	113	30,000	240	19.14	0	0	43	Good condition
6	5	107	35,000	242	19.43	0	0	30	Good condition
7	35	20	50,000	151	16.87	0	0	10	Good condition
8	18	115	50,000	272	14.62	0	0	29	Good condition
9	26	38	50,000	300	19.30	0	?	45	Fair condition. Still 15% under weight.
10	15	8	105,000	218	18.72	-	-	30	Fair condition. 10% underweight.

capsule after dilution with cottonseed oil. Vitamin A was administered along with D to study its possible protective effect (see Table 33 for administration protocol). The purified diet fed to the dogs following weaning and throughout the experiment contained a concentration of Ca (1.0%) and P (0.73%) comparable to that in the milk solid diet given to infants treated by single or repeated massive doses of D (for diet composition, see original paper). At the termination of the experiment the animals were sacrificed and the tissues examined. Some of the observations were:

a. All the dogs receiving excess D (10,000 IU/kg BW/day), exhibited some toxic symptoms.
b. The dogs overdosed with tuna liver oil showed less toxicity, and the ones given dehsterol more toxicity than those given irradiated ergosterol.
c. All the overdosed dogs showed: stunted height and weight; some degree of abnormal calcification in nearly all the soft tissues; excessively mineralized long bones; and shafts increased in thickness. The teeth were small with deformed roots, pulp stones and inflamed gum tissue.
d. The symptoms of hypervitaminosis induced in one premature female dog were more severe than in the other animals, and her recovery during rest periods was delayed and incomplete.
e. All the dogs given excess D had a raised serum Ca level.
f. In the most severely affected animals the proportion of excess phosphorus to excess Ca was 2:1 in the kidneys, heart and femoral muscles, and 0.5:1 in the lungs and stomach.
g. The two dogs which were relieved of medication after receiving it for 109 and 123 days respectively, showed no repair of damaged teeth and jaws even though their appetite and growth improved. When sacrificed, their stomachs, lungs and kidneys showed excessive Ca retention.

The authors concluded from their observations that:

a. The cumulative effect of a repeated moderately excessive dose of D was not as severe as the effect of one massive dose. According to the authors a single massive dose was the usual method for treatment or prophylaxis of infantile rickets.
b. A question may be raised concerning the unusual susceptibility of premature infants to hypervitaminosis, judging from the extremely

Table 53

Amounts and Sources of Vitamins D and A Fed Young Dogs (2547)

Group	Dog	Vitamin D per kg per day (IU)	Vitamin A per kg per day (IU)	Total Period on diet (days)	Number of excess daily doses given	Total vitamin D given (IU x 1000)
1. Optimum vitamin D and vitamin A	1♂	72, tuna liver oil	800, tuna liver oil and halibut liver oil	302		161
	5♂			296		153
	9♀	72, irradiated ergosterol	800, halibut liver oil	296		119
2. Optimum vitamin D and excess vitamin A	2♂	72, halibut liver oil	10,000, halibut liver oil	302		161
	6♂			296		153
	8♀	72, irradiated ergosterol	10,000, carotene in oil	296		119
3. Excess vitamin D and excess vitamin A	11♀	10,000, tuna liver oil	10,000, tuna liver oil and halibut liver oil	296	236	15,500
	3♀	10,000, irradiated ergosterol	10,000, halibut liver oil	312	236	14,400
	7♂			296	236	18,900
	13♂*	10,000, irradiated ergosterol	10,000, shark liver oil	360	147 in 188 days	12,000
	12♂*	10,000, delsterol	10,000, shark liver oil	360	127 in 188 days	9,500
4. Excess vitamin D and optimum vitamin A	4♀	10,000, irradiated ergosterol	800, halibut liver oil	312	236	15,530
	10♀			296	236	12,080

* These dogs were relieved of medication and allowed a recovery period of 146 days.

adversely affected premature animal in their experiment.

c. The apparently greater toxicity resulting from the moderately excessive D given in their experiment as compared to that described in previous reports was probably due to the greater Ca content of the diet, the youth of the animals and the longer period of medication.

F. Ruminants

1. In 1964, Fell et al. (1766) investigated possible pathological effects of a high dose of D_3 to sheep. Eleven months before slaughter, seven Blackface ewes were administered a single i.m. injection of one million units of D_3 in 2 ml ethyl oleate. Histological examination showed non-calcified arteriosclerotic intimal lesions in two of the sheep. The other five had a mild, diffuse, medial fibrosis of the aortic wall. Since the authors postulated that the arteriosclerotic lesions were due to vitamin D toxicity, they made the following similar study to check their observation.

Forty, one-year old healthy Blackface ewe hoggs were divided into three groups and injected with D_3 as shown in Table 34.

Table 34

Injections of D_3 to Sheep (1766)

Group	No. of ewes	Intramuscular injection	Slaughter: time after injection 2 mos.	4 mos.	6 mos.	8 mos.
1	16	1,000,000 units vitamin D_3 dissolved in 2 ml ethyl oleate (Robert Young & Co. Ltd.)	4	4	4	4
2	8	500,000 units vitamin D_3 in 1 ml ethyl oleate	-	4	-	4
3	16	2 ml ethyl oleate	4	4	4	4

Microscopic examination showed diffuse lesions in the aorta of all the animals injected with 1,000,000 units of D_3. No lesions were found in the heart and lung. Localised arteriosclerotic lesions were found in a number of both the treated and control animals. The authors believed the diffuse changes in the arterial wall were genuine pathological effects of the D_3 treatment.

2. In 1965, Packett and Coburn (4415) found that D added to the feed of yearling sheep enhanced urolithiasis. A diet for fattening lambs was supplemented with D_3, 200 IU per lb of feed and fed to 15 Texas-bred Rambouillet wethers.

Calculi were found mainly in the bladder of 12 of the lambs. These were usually fine, chalky particles but some stones weighing several milligrams were found. Vitamin D produced the highest incidence of urolithiasis of six experimental dietary supplements.

G. Monkeys

In 1958, Kent et al. (3104) reported the clinical and pathological observations made on a monkey colony accidentally fed excessive amounts of Ca, P and D for about three months. Owing to an error in food manufacture, 558 monkeys (Macaca mulatta) were fed a diet which included 162,000 U.S.P. units of D per animal per day, plus 3.5 g of Ca and 2.9 g of P daily. Once the error was discovered, the animals were placed on a low D diet.

The observations were:

a. A greater than usual incidence of upper respiratory infection and diarrhea.

b. Weight loss starting shortly after the inception of the high D diet.

c. An appreciable decrease in erythrocytes and hemoglobin during and after the period of excess D intake.

d. No changes in the long bones.

e. Characteristic lesions consisting of calcium and iron deposits were found more consistently in kidneys than in any other tissue.

f. Lesions were also found in the lungs but not as regularly as in the kidneys. These appeared in the lungs of 23 of 39 animals dying between the 55th to 208th day.

g. Cardiac lesions were noted in nine of the animals which died between the 55th and 86th days. (Table 35 summarizes the distribution of calcium found in the kidneys, lung and heart).

h. Mineral deposits were also found in the aortas of 12 of 34 animals dying between the 55th and 140th days.

i. Next to renal involvement, the earliest and most common evidence of calcification was found in the salivary glands of 27 out of 43 animals dying between the 47th and 226th days.

j. The first lesions appeared after 28 days on the diet containing excessive D. Really severe lesions were noted after 55 days.

k. In about one month after termination of the high D diet, the surviving animals appeared in good health. After a year few lesions were seen.

H. Humans

1. In 1937, Steck et al. (5512) observed the effects of massive doses of D given to humans. The experiment was carried out with 773 subjects ranging

Table 35

Distribution of Calcium Deposits in Heart, Kidneys, and Lungs (3104)

Organ	Animal Number									
	213+	551	54+	C-8	55+	X-7	133	61+	86+	165+
Heart										
Left ventricle										
Apex	++*	o	+	++++	++	+	+	**	+	o
Base	+++	+	o	+++		+	o	+	+	
Middle	++++	++	+	++++	++	+	+	+	+	o
Right ventricle										
Apex	o	o	o	+++	o	o	o	o	o	o
Base	+	o	o	++	o	o	o	o	o	o
Left atrium	+	o	o	++	++	o	o	o	o	o
Right atrium	o	o	o	o	o	o	o	o	o	o
Kidney										
Superior pole										
Right	+++	+++	++	++	+++	+++	+++	+++	+++	+
Left	+++	++++	++	++	+++	+	+++	+++	+++	+
Inferior pole										
Right	+++	+++	+++	++	+++	+++	+++	++	+++	+
Left	+++	++++	+++	++	+++	+	+++	+++	+++	+
Middle										
Right	+++	++++	+++	++	+++	+++	+++	+++	+++	+
Left	+++	+++	+++	++	+++	+	+++	+++	+++	+
Lung										
Upper lobe										
Right	++++	+++	+++	++	++	++	+	++	+	o
Left	++++	+++	+++	++	++	++	+	++	+	o
Middle lobe										
Right	+++	+	++	++	++	++	+	++	+	o
Left	++++	+	++	++	++	+	+	++	+	o
Lower lobe										
Right	++++	++	++	++	++	++	+	++	+	o
Left	++++	++	+++	++	++	++	+	++	+	o

*The quantity of calcium was estimated on the basis of the amount of black precipitate in the von Kossa stained sections: ++++ = most severe calcification noted in a given organ, +++, ++, + = 75, 50, 25 percent of the maximum.
**Tissue not available for examination.

in age from 17 to 76 who were administered daily doses of more than 100,000 units of D for periods ranging from seven days to five years. The status of the test subjects is shown in Table 36 and the incidence of the toxicity at each dosage range is shown in Table 37.

The authors noted that they determined from additional statistics the order of decreasing susceptibility among the different groups of patients was: arthritis, normal subjects, hay fever alone, hay fever with asthma, tetany.

Some observations were:

a. The shortest period of administration producing toxicity in the group on 3,000-5,000 units/kg/day was 87 days (see Table 37).

b. In the group on 6,000-7,000 units/kg/day, the shortest period for toxicity to develop was 60 days (see Table 37).

c. The authors could not reconcile their findings of a low incidence of toxicity with those of another study around the same time reporting 100% toxicity in 22 human subjects administered massive doses of D.

d. Older subjects were not easily made toxic but when toxicity occurred they recovered less well and developed sensitivity to D.

The authors cautioned that massive doses of D should only be taken under a doctor's supervision and discontinued at the first signs of toxicity. (See Biological Data pp.88-94 for a similar experiment by these authors with dogs.)

2. In 1943 Ziskin et al. (6376) took dental radiographs of eight patients suffering from rheumatoid arthritis before and after administration of 300,000 IU of D (Ertron) daily. The dosage schedule is summarized in Table 38. No appreciable change with respect to pulp stone formation was seen. For a similar study with rats by the same researchers see p. 66.

3. In 1948 Cogan et al. (1074) reported five cases of vitamin D poisoning.

a. In the first case the patient (male, 27 years old) had taken 500,000 units of D per week for four years. Serum Ca levels were elevated (12.6 mg), the npn level was 52 mg and P_i 4.3 mg, eyes showed a typical band keratopathy, and after death autopsy revealed nephrocalcinosis.

b. In the second case the patient (female, 39 years old) had taken 100,000 units of D daily for five years. Serum Ca levels were found to be 10.2 to 14.4 mg, npn 50 to 70 mg, and P_i 4.8 to 6.3 mg. The eyes showed superficial corneal opacities and the conjunctivae showed numerous, presumably calcific, fine opacities.

Table 36. Status of 773 Human Subjects Who Received More than 100,000 Units of D Daily (5521)

	Male			Female		
	Total No.	No. Toxic	Percent	Total No.	No. Toxic	Percent
Postoperative tetany	2	0	0	15	4	26.4
Hay fever and asthma	178	13	7.3	322	24	7.4
Arthritis	43	5	11.6	107	11	9.4
Miscellaneous	12	1	8.3	23	3	13
Normal subjects	63	1	1.5	8	1	12.5
	298	20	6.7	475	43	9

Total subjects 773
Number toxic 63
Percent toxic 8

Table 37

Incidence of Toxicity at Each Range of Dosage (5521)

Units/kg/day	No.	No. Toxic at any Stage	Percent Toxic
1,500-3,000	5	5	100
3,000-5,000	555	25	4.5
6,000-7,000	123	18	14.6
8,000-15,000	70	11	15.7
15,000-25,000	16	3	18.8
25,000-35,000	4	1	25
	773	63	8+

Table 38

Vitamin D* Dosage of Rheumatoid Arthritic Patients (6376)

Patient	Age	Sex	Total Unit Dosage	Total Dosage Period (Days Between Radiographs)
J.W.	33	Female	41,700,000	153
H.S.	47	Female	68,250,000	231
C.P.	71	Female	29,150,000	103
V.B.	55	Female	34,550,000	121
E.W.	29	Female	62,250,000	218
I.R.	61	Female	48,950,000	169
L.T.	48	Male	63,750,000	219
I.F.	48	Female	67,250,000	230

Note: In 3 cases the first set of radiographs was taken a few days after instituting therapy.

*Ertron

c. In the third case the patient (female, 70 years of age) had taken 150,000 units of D daily for six months along with large amounts of milk. Her serum Ca level was 11.7 to 13.9 mg, npn 41 to 66 mg and P_i 4.3 to 5.3 mg. The eyes showed a definite type of opacity in the palpebral fissure extending to 2 or 3 mm from the limbus.

d. In the fourth case the patient (male, 65 years old) had taken 300,000 units of D daily for two to three years. His serum Ca value was 11.6 to 15.2 mg, npn 45 mg and P_i 4.3 to 5.2 mg. The eyes showed a typical band of superficial opacities in the palpebral fissure of both corneas.

e. In the fifth case the patient (female, 55 years old) had taken 300,000 units of D daily for two-and-a-half months along with large quantities of milk. The serum Ca level was found to be 13.4 mg, npn 62 mg and P_i 5.7 mg. Roentgenograms showed calcification of soft tissues about the hands, shoulders, back and knees. Examination of the eyes showed bilateral and symmetric opacification of the nasal and temporal portions of the paralimbal cornea in the palpebral fissure.

The authors considered that the npn and P_i levels in the blood indicated renal insufficiency in all of the patients. All of the patients were hypercalcemic and had band keratopathy. The doses of D taken ranged from 100,000 to 500,000 units daily for periods from two-and-a-half months to five years.

4. In 1948 Debré (1344) reported on 21 cases, two fatal, of D overdosage in children. In both fatal cases (20 and 16 months), the infants had received 11,200,000 and 18,200,000 units of D_2 respectively. When 3,000,000 to 6,000,000 units were given the toxicity was less severe. In one non-fatal case, the child had hemiplegia with a serious mental deficiency. In two other cases in which there was serious nervous trouble, the blood pressure was extremely high.

The author concluded from his clinical observations of all his cases that renal and cerebral impairment were the two main dangers of D toxicity in children.

5. In 1948 Howard and Meyer (2793) reported the cases of 11 patients intoxicated by administration of D preparations. One patient became hypercalcemic after only 14 days administration of 300,000 IU calciferol. The patients ranged from 33 to 68 years old, five males and six females. The doses given ranged from 150,000 to 600,000 IU. Toxic symptoms were seen anywhere from two to 18 months after onset of therapy. Even though the patient receiving the highest

dose, 600,000 IU, became ill earliest, there was no other correlation between dose size and appearance of symptoms. (See Table 39 for details of the case histories).

The authors noted the outstanding symptoms of the D intoxication to be fatigue, weight loss, anorexia, and vomiting. The outstanding clinical signs were impairment of renal function and degenerative lesions with vicarious calcification such as "band keratitis". In all cases npn (46-100 mg per cc) and serum Ca (12.4 to 15.1 mg per 100 cc) were elevated. The authors pointed out that there was a wide variation in sensitivity of patients to the various D drugs administered: Ertron, Davitin, Dalsol, Deltalin, Darthronal, all of which contained 50,000 IU D per capsule.

6. In 1949, Stanbury (5488) reported a case of D poisoning. A 36 year old woman with rheumatoid arthritis received 100,000 units of D daily for three weeks, seven months before hospitalization. She had also been taking for a month four tablets daily of a vitamin preparation containing 25,000 units of D per tablet prior to admission. Her blood Ca was found to be considerably elevated, 13.7 mg per 100 cc. The P was 3.9% and the APase 3 units %. Band keratitis was found in her eyes.

X-rays showed considerably more calcification of the cartilaginous structure, particularly in the costal cartilage and the thyroid cartilages, than could be expected for a 36-year old person. The hypercalcemia was attributed by Dr. Bernard G. Stall, the attending physician, to the 100,000 units of D taken by the woman.

It was also considered surprising that the effects of the poisoning continued as long as two weeks after the medication was stopped, thus indicating that the effect was not transitory but that the damage could continue long after the drug was stopped.

One physician, Dr. Marian W. Ropes, recommended that since D is toxic to a fair percentage of people but the symptoms are not recognized until damage is done, no one should take excessively high D doses.

7. In 1950 Ryde and Richmond (2832) reported the effect of large doses of D given to a 12-year-old with rheumatoid arthritis over a period of five and one-half years. The dosage given daily by mouth was 100,000 to 150,000 USP units of a proprietary irradiated ergosterol.

Table 39. Case Histories of Patients with D Intoxication (2793)

Case	Presenting Features	Serum values after omission of medication: initital and follow-up								Initial Urinalysis		
			NPN mg %	Ca mg %	P mg %	CO_2 mEq/L	Cl mEq/L	Total Prot. Gm %	Alb. Gm %	Glob. Gm %	Alb.	RBC	Casts
Case 1. J.O. JHH#386002 W M 56	Weight loss-20 lbs. in 6 mos., Anorexia weakness and fatigue Nausea and vomiting 2 wks. No genito-urinary complaints. No anemia - hgb. 13.1 Gm.	Initial	52	13.9	4.0	--	--	6.2	4.2	2.0	2+	0	0
		After 3 wks.	--	11.6	4.0	--	--	--	--	--	2+	0	
Case 2. J.S. JHH#419594 W M 54	Weight loss-40 lbs. in 6 mos., Anorexia weakness and vague abdominal pain. Nausea and vomiting 3 mos. Nocturia (3X) for 4 mos. Anemia-hgb. 9.0 Gm. RBC 3.61; Ht. 28 (microcytic hypo-chromic)	Initial	89	13.6	4.6	29.2	101.6	6.7	4.7	2.0	1+	0	Occ. hyal. & gran.
		After 2 wks.	27	11.4	3.1	27.6	100.0	6.5	--	--	1+	0	Occ. hyal. & gran.
		After 2 mos.	33	10.4	3.0	--	--	7.1	--	--	0	0	0
Case 3. W.C. JHH#421206 W F 65	Weight loss-30 lbs. in 3 mos., Anorexia weakness and indi-gestion 2-3 mos. Nausea and vomiting 2 mos. Nocturia 2-3X. Severe backache and joint pains 6 wks. Slight anemia hgb. 12.0 Gm. RBC 4.4. Ht. 36 (normo-cytic normochromic)	Initial	73	15.1	5.2	28.7	98.5	6.6	4.6	2.0	1+	0	Occ. hyal. cast
		After 3 wks.	43	12.7	3.6	--	--	7.0	--	--	0	0	

Table 39 (cont.)

Case	Presenting Features	Serum values after omission of medication: initital and follow-up								Initial Urinalysis		
			NPN mg %	Ca mg %	P mg %	CO_2 mEq/L	Cl mEq/L	Total Prot. Gm %	Alb. Gm %	Glob. Gm %	Alb.	RBC	Casts
Case 4. A.W. JHH#194939 W M 59	Weight loss-30 lbs. in 1 yr. Weakness and fatigue. Nausea and vomiting (periodic) 4 mos. General pruritis 4 mos. Polydipsia, polyuria and nocturia (4-5X) 4 mos. Anemia - hgb. 8.6 Gm. RBC 3.2 Ht. 23 (microcytic hypochromic)	Initial	62	14.3	4.6	29.5	101.6	7.0	4.3	2.7	1+	0	Occ. gran. cast
		After 1 mo.	62	13.9	4.1	--	--	7.4	--	--	1+	0	0
		After 3 mos.	68	13.3	4.2	--	--	7.4	--	--	2+	0	0
		After 10 mos.	46	12.2	2.5	25.4	108.1	6.9	--	--	1+	0	0
		After 16 mos.	50	10.6	2.6	22.8	--	7.9	--	--	0	0	0
Case 5. F.H. JHH#168084 W F 40	Weight loss-25 lbs. in 6 mos. Weakness and fatigue. Nausea and vomiting 6 mos. No genito-urinary complaints. Anemia hgb. 10.5 Gm. RBC 4.31. Ht. 34 (microcytic hypochromic)	Initial	46	13.6	3.9	25.4	107.0	7.1	4.5	2.6	0	0	0
		After 4 mos.	40	10.1	2.2	27.6	96.4	7.4	--	--	0	0	0
Case 6. L.S. JHH#414730 W F 54	Slight weight loss 4 lbs. Marked indigestion, nausea and vomiting. Headaches weakness and fatigue Nocturia (4-6X) 1 yr. Anemia - hgb. 8.8 Gm. RBC 3.63. Ht. 27 (microcytic hypochromic)	Initial	57	14.9	3.5	28.7	107.0	7.0	--	--	0	0	Occ. hyal. cast
		After 3 wks.	45	11.0	3.2	--	--	7.0	--	--			
		After 3 mos.	39	10.8	3.2	--	--	7.0	--	--			
		After 6 mos.	39	11.1	3.6	--	--	7.1	--	--			

Table 39 (cont.)

Case	Presenting Features	Serum values after omission of medication: initital and follow-up	NPN mg %	Ca mg %	P mg %	CO_2 mEq/L	Cl mEq/L	Total Prot. Gm %	Alb. Gm %	Glob. Gm %	Initial Urinalysis Alb.	RBC	Casts
Case 7. M.D. JHH#446602 W F 68	Slight weight loss 5 lbs. No G.I. symptoms. Fatigue and weakness. General pruritis during vit. D period. Slight polyuria; left kidney removed 18 mos. previously and was polycystic (other kidney said to be normal). Anemia-hgb. 9.2 Gm. RBC 3.33 (normocytic normochromic)	Initial	53	13.6	3.8	--	--	6.8	4.9	1.9	Tr	0	0
		After 2 mos.	43	11.5	3.6	—	—	6.8	--	--	0	0	0
Case 8. J.B. Patient of Dr. W.A. Baetjer WM 45	Weight loss-10 lbs. 6 mos. No nausea or vomiting. Fatigue and weakness, marked frequency, polyuria polydipsia, nocturia 6 mos. Anemia - hgb 11.1 Gm. RBC 4.2. Ht. 31 (microcytic hypochromic)	Initial	75	14.9	3.6	--	--	6.5	—	--	0	Occ.	0
		After 10 days	56	13.2	3.6	—	—	5.8	—	—			
		After 4 days	35	12.9	2.1	—	—	6.9	—	--			
		After 11 mos.	49	10.7	2.3	--	--	6.6	3.9	2.7			
Case 9. W.K. Patient of Dr. C.R. Austrian W M 62	No weight loss. Anorexia, fatigue and weakness. Intractable nausea 2 wks. Nocturia and frequency. Anemia hgb. 70%. RBC 3.76 (normocytic normochromic)	Initial	106	12.4	3.0	--	--	—	—	—	Tr	1-3	0
		After 3 wks.	37	9.4	4.0	--	--	--	--	--	—	--	--
		After 4 mos.	30	8.9	3.0	--	--	7.6	4.2	3.4	—	--	--
		After 11 mos.	37	10.0	3.0	—	—	7.2	4.3	2.9	0	0	Occ. gran.

Table 39 (cont.)

Case	Presenting Features	Serum values after omission of medication: initital and follow-up								Initial Urinalysis		
			NPN mg %	Ca mg %	P mg %	CO_2 mEq/L	Cl mEq/L	Total Prot. Gm %	Alb. Gm %	Glob. Gm %	Alb.	RBC	Casts
Case 10. D.M. Patient of Dr. J.H. Trescher W F 33	No weight loss. Nausea and vomiting 2 yrs. Fatigue and weakness. Sore eyes for 2 yrs. Nocturia 6 mos. Anemia - hgb 10.4 Gm. RBC 3.6. Ht. 30 (normocytic normochromic)	Initial	53	13.7	5.0	--	--	6.2	4.8	1.4	Tr	0	0
		After 1 mo.	--	14.4	5.6	--	--	7.0	--	--	--	--	--
		After 3 mos.	--	13.5	4.5	--	--	7.0	--	--	Tr	0	0
		After 6 mos.	--	9.2	2.6	--	--	--	--	--	--	--	--

Table 39 (cont.)

Case	B.P. mm. Hg	Vit. D preparation and dosage	Length of time vit. D taken before symptoms appeared	Duration of symptoms when diagnosis established	Renal function: initial and follow-up
Case 1. J.O. JHH#386002 W M 56	135/85	150,000 units daily for 4 mos. "Ertron"	3 1/2 mos.	16 days	Fishberg: 1.010 PSP: 28% Urea clearance: 38% & 20%
Case 2. J.S. JHH#419594 W M 54	160/90 In 2 wks. fell to 125/70	300,000 units daily for 6 mos. "Davitin"	4 mos.	2 mos.	Fishberg: 1.012 PSP: 15% After 2 wks., PSP: 45% After 2 mos., PSP: 61% Fishberg: 1.014 Urea clearance: 34% & 52%
Case 3. W.C. JHH#421206 W F 65	158/88 In 3 wks. fell to 120/80	200,000 units daily for 18 mos. "Deltalin"	15 mos.	2 mos.	PSP: 15% Fishberg: 1.010 Urea clearance: 16% & 11% After 3 wks., PSP: 22% Fishberg: 1.012
Case 4. A.W. JHH#194939 W M 59	110/70	500,000 units daily for 18 mos., stopped 4 mos. before admission "Ertron"	18 mos. Calcium deposits noticed in skin at 13 mos.	4 mos.	PSP: 10% Fishberg: 1.018 Urea clearance: 14% & 26% After 3 mos., PSP: 16% After 10 mos., PSP: 33% Urea clearance: 40% & 42% After 16 mos., PSP: 18%
Case 5. F.H. JHH#168084 W F 40	122/78	Dosage not accurately determined. Approx. 150,000 units daily for 12 mos. "Ertron" & "Darthronal"	12 mos.	6 mos.	PSP: 80% Fishberg: 1.026 After 4 mos., PSP: 80%

Table 39 (cont.)

Case	B.P. mm. Hg	Vit. D preparation and dosage	Length of time vit. D taken before symptoms appeared	Duration of symptoms when diagnosis established	Renal function: initial and follow-up
Case 6. L.S. JHH#414730 W F 54	170/85 In 3 wks. fell to 140/80	400,000 units daily for 12 mos. "Davitin"	10 mos.	6-8 wks.	PSP: 25% Fishberg: 1.014 After 3 wks., PSP: 28% After 3 mos., PSP: 55% After 6 mos., PSP: 67% Fishberg: 1.022
Case 7. M.D. JHH#446602 W F 68	120/76	600,000 units daily for 2 mos. "Dalsol"	6 wks.	2 wks.	PSP: 5% Fishberg: 1.010 After 2 mos., PSP: 20% Fishberg: 1.012
Case 8. J.B. Patient of Dr. W.A. Baetjer W M 45	120/80	300,000 units daily (irreg.) for 18 mos. "Ertron"	12 mos.	6 mos.	PSP: 9% Fishberg: 1.010 After 2 wks., PSP: 40% Fishberg: 1.011 After 11 mos., PSP: 58% Fishberg: 1.018
Case 9. W.K. Patient of Dr. D.R. Austrian W M 62	204/90	300,000 units daily for 5 mos. "Deltalin"	4 1/2 mos.	2 wks.	Fishberg: 1.011 Urea clearance: 21% & 16% After 11 mos., PSP: 57%
Case 10. D.M. Patient of Dr. J.H. Treacher W F 33	120/80	200,000 to 400,000 units daily for 4 yrs. "Ertron"	2 yrs. Soreness of eyes noted first	2 yrs.	No initial renal function test. After 3 mos., PSP: 22% After 7 mos., PSP: 25% After 9 mos., PSP: 45%

Table 39 (cont.)

Case	Metastatic calcification	Length of time for symptoms to subside following withdrawal of vit. D
Case 1. J.O. JHH#386002 W M 56	None found.	Improved considerably during hospital stay. No further follow-up.
Case 2. J.S. JHH#419594 W M 54	Cornea & conjunctivae only. Recession of crystals in 1 mo. Band keratitis less but persisting.	Practically complete return to well-being in 2 wks. Nausea persisted for 2 wks. After 2 mos., all symptoms gone; weight gain, return of alertness & strength. Moderate anemia persists.
Case 3. W.C. JHH#421206 W F 65	Cornea & conjunctivae only. No follow-up examination.	Marked symptomatic improvement during her 3-wk. hospitalization. Vomiting stopped immediately, backache disappeared; weight gain of 3 lbs. No further follow-up obtained.
Case 4. A.W. JHH#194939 W M 59	Cornea & conjunctivae, nail beds, lips, skin of hands and face. Recession of calcifications in 16 mos.	Symptoms minimal. Serum calcium normal in 20 mos. Renal insufficiency persists.
Case 5. F.H. JHH#168084 W F 40	Conjunctivae only. Recession of conjunctival crystals in 4 mos.	Improvement very definite after first admission. At 4 mos. follow-up there were no complaints.
Case 6. L.S. JHH#414730 W F 54	Conjunctivae only. Recession of conjunctival crystals in 6 mos.	Improvement not immediate but by 3 mos. nocturia had lessened; gastro-intestinal symptoms were abated except for slight p.c. discomfort; anemia markedly improved. Exacerbation of arthritis 4 mos. after vit. D stopped.
Case 7. M.D. JHH#446602	Cornea & conjunctivae only. No follow-up examination.	General well-being; anemia and pruritis all greatly improved 2 mos. after vit. D was stopped.

there was a reversible increase in bone density with severe calcinosis and renal failure.

17. In 1962 Nigrin et al. (4269) reported 11 cases of refractory hypophosphatemic rickets all of which had been treated with large doses of D. Histological examination showed patchy distribution of intratubular Ca deposits either in the form of obstructing casts or as small particles and dilatation of tubules. Calcification was found in all cases except one. The authors concluded that irreversible renal changes could result with large doses of D, despite constant surveillance.

18. In 1963 Cuthbertson (1257) reported the D content of the sera of 11 children with idiopathic hypercalcemia as compared to plasma samples from healthy children. The results showed that the D levels for patients with the disease, either in mild or severe form, did not differ from each other or from those of the controls.

19. In 1964 Black (0589) reviewed the relationship of D to idiopathic hypercalcemia and compared the clinical pathology resulting from D overdosage to that of idiopathic hypercalcemia noting similarities. Three cases in which overdosage with D produced a clinical picture similar to that of severe hypercalcemia are summarized in Table 41. The significance of D activity in the serum is discussed. Some data from case histories are summarized in Table 42.

In conclusion the author noted that consideration of the facts concerning D toxicity is necessary in examining national policies concerned with the fortification of milks with vitamin D, as well as in the study of the current use and abuse of D by the medical profession and the general public.

20. In 1964, DeLuca (1320), reported on 12 infants between the ages of 7 and 30 months intoxicated by D overdosage. The cases are summarized in Table 43. The laboratory tests confirming the clinical signs of D intoxication are summarized in Tables 43, 44 and 45.

In 10 of the 12 cases discussed, a clearcut picture of renal tubular acidosis was established. The doses administered were from 48,000 IU to 200,000 IU daily with a total dosage between 1,200,000 and 10,000,000 IU. The authors considered the daily dose more significant with respect to effect on the clinical picture than the total dose. The authors noted the presence

Table 39 (cont.)

Case	Metastatic calcification	Length of time for symptoms to subside following withdrawal of vit. D
Case 8. J.B. Patient of Dr. W.A. Baetjer W M 45	Cornea & conjunctivae only. No follow-up examination.	In 2 wks. patient had marked return to well-being. Especially notable was return of strength. Arthritis remained quiescent. Anemia persisted for a while but was completely gone at 11 mos.
Case 9. W.K. Patient of Dr. D.R. Austrian W M 62	None found.	In 4 days nausea had disappeared. In 1 wk. patient was asymptomatic. Anemia persisted but gradually improved and was gone at 11 mos. There was return of arthralgia and objective joint swelling within 10 days after vit. D stopped.
Case 10. D.M. Patient of Dr. J.H. Trescher W F 33	Cornea & conjunctivae. Conjunctival involvement still present at 5 mos. No further follow-up.	Within 1 mo. dramatic improvement in well-being. Joints bothered patient less. Eyes much better with loss of soreness. Anemia gradually improved in 9 mos.

Some of the symptoms of D intoxication observed were anorexia, vomiting, polydipsia, polyuria, and nocturia; calcification of blood vessels, periarticular soft tissue, conjunctivas, and corneas; hypertension, hypertensive retinopathy, and renal insufficiency.

There was some amelioration of these symptoms after discontinuance of D therapy but after two years, renal function showed no significant improvement. The authors considered this was indicative of irreversible kidney damage.

8. Adams (0025) reported the case of a 40-year old man with uremia caused by D poisoning. The patient appeared to have severe chronic renal impairment but none of the symptoms usually associated with renal disease. Questioning of the patient brought out that he had been taking 200,000 units of D daily for six months which had been prescribed for shoulder pain.

The main symptoms were: increased fatigue and weakness, polydipsia and polyuria, nitrogen retention and a high blood calcium. The patient improved after the D was stopped. The author noted that the case was reported in order to emphasize the danger of D overdosage and its possible cause of uremia and hypercalcemia.

9. In 1951 Chaplin et al. (0972) reviewed 111 cases of D intoxication in the literature and reported on seven additional cases at their hospital. All seven patients had taken D preparations (over 50,000 IU to 300,000 IU daily) before admission for periods ranging from three weeks to six years.

The authors consistently found "band keratitis" and metastatic calcium cysts. They expressed concern about the intake of large quantities of D by vitamin-habituated individuals as well as the need for medical surveillance when massive doses of D are prescribed.

10. In 1951 Medical Grand Rounds (5487) presented the case of a woman, 59 years old, with a mental disorientation along with other symptoms. For the previous six weeks to two months, she had been taking a vitamin preparation after meals containing 50,000 units of D per capsule for a total of 150,000 units per day. Other symptoms were: elevated serum Ca, alkalosis, high CO_2, high npn and serum P, corneal opacification and diminished renal function. One of the discussants, Dr. Allan Butler, considered most of the picture to be typical of D intoxication. The patient improved mentally and generally after medication was stopped.

11. In 1954 Creery (1213) described 16 cases of idiopathic hypercalcemia. An estimate of the D intake from diet and supplementation showed that: two infants had about 700 IU daily, six had 1100 to 1600 IU daily, seven had 1800 to 2400 IU daily and one had 3200 along with a high Ca intake. Table 40 summarizes the cases. In all but two, initial examination of the serum showed a Ca level above 12 mg per 100 ml, and in some cases it was as high as 16 to 17 mg per 100 ml. The blood urea was almost always elevated.

Radiography showed in 12 cases transverse bands of increased density at the metaphyseal margins of the lower ends of the radius and ulna in the wrists with a similar appearance in the metacarpals in some instances. In eight cases, a similar appearance was noted in the femur and tibia. No calcification of the kidneys was observed.

The author noted that the only constant etiological factor was a high Ca intake from early infancy, usually associated with a more than adequate amount of D in the diet.

12. In 1957, Scharfman and Propp (5061) reported the cases of four patients with normocytic normochromic anemia which was resistant to treatment who had taken 50,000 to 150,000 units of vitamin D daily for years. They found these patients all had symptoms of D intoxication and some degree of renal impairment. The authors concluded that anemia was almost always present with D excess.

13. In 1957 Bongiovanni et al. (0656) described three cases of "idiopathic hypercalcemia of infancy, with failure to thrive". According to them, the syndrome had first been described by Lightwood in 1932 and was so named by him in 1952 after Fanconi and Butler had presented further cases. The syndrome was common in Britain, but rare in America.

a. The authors gave a description of the syndrome (see paper), emphasizing increased Ca retention and impaired kidney function.

b. They inferred D to be a major cause. Either there were hypersensitivities to ordinary doses, or recommended doses were too high

The authors' own cases each had received over 1000 IU/day, and they recommended in summary that 400-500 IU/day should not be exceeded.

14. In 1959 Smith et al. (5381) reported a case of prolonged hypercalcemia. A five-year old patient was hospitalized for severe idiopathic hypercalcemia first seen at ten months in a mild form. Vitamin D intake was about 1400 IU per day until 18 months, 1500 IU per day until 5 years and 4400 IU per day one

Table

Summary of the Cases (1213)

Case no.	Age at onset (mos.)	Age at recognition (mos.)	Duration to clinical recovery (mos.)	Highest serum-calcium (mg. per 100 ml.)	Highest blood-urea (mg. per 100 ml.)	Follow-up				
						Duration (mos.)	Last known serum-calcium (mg. per 100 ml.)	Last known blood-urea (mg. per 100 ml.)	Stature	Other remarks
1	1 1/2	17	18	16	35	18	10.3	30	Average	Mentally retarded (not connected with illness ?)
2	3	13	13	15.9	121	18	12.8	49.7	Below Average	Secondary rise in serum-calcium age 27 months
3	9	12	4	13	33	12	8.7	33	Below Average	..
4	4	7	11	16.8	92.6	9	12	45	Average	..
5	Birth	17	22	12.3	39	6	11.2	30.3	Average	..
6	2	13	16	14.2	61	3	11.7	43	Average height Underweight	Urinary infection at follow-up
7	8	12	8	13.8	43	5	10.6	46.4	Slightly below average	Anaemic at follow-up (62%)
8	7	14	14	12.5	46	1	11	28.5	Average height Underweight	Recurrent tonsillar sepsis
9	3 1/2	7	11	14.5	96	6	11.7	43	Average height Underweight	Anaemic at follow-up (56%) ..
10	1 1/2	6	7	13.1	52	6 1/2	11.7	37.5	Below average	
11	5	11	..	14.5	80	..	..	..	..	Lost sight of
12	7 1/2	7 1/2	1	14.7	78	3	12.3	32	Average	..
13	5	9 1/2	..	14.4	98.5	..	..	..	..	Remains in active stage
14	6	11	6	14.1	46.4	1	11.4	36.4	Average height Underweight	..
15	8	10	..	17.3	100	..	..	..	..	Remains in active stage
16	5	8	..	17.5	105	..	..	..	..	Remains in active stage

month before hospitalization. The D levels in the serum were 23 units/ml of plasma (normal value is 0.66 to 1.65 units/ml). Roentgenograms showed demineralization of the long bones. After discontinuation of supplemental D and a diet low in Ca, the concentrations of Ca and D in the serum gradually returned to normal over an 18 month period. The impairment of renal function, an intelligence defect and a perceptive hearing defect showed no improvement.

15. In 1960 Lehrer and Levitt (3453) reported two cases in which D intoxication was associated with an organic mental syndrome. Two patients (females aged 48 and 67) had taken 100,000 IU daily for six weeks and six years respectively. The younger woman had an elevated blood Ca and was lethargic, confused and disoriented. When placed on a low Ca diet the confusion gradually disappeared. The older woman showed severe memory loss, disorientation and confusion becoming dull and apathetic with symptoms of nervous system dysfunction. Serum Ca was found to be 15.8 mg %. X-ray examination showed considerable demineralization of the long bones, pelvis and hands. Along with taking the 100,000 units of D, this patient ate large quantities of sour cream.

After being placed on a low Ca diet, the patient's Ca level fell to 12.0 mg %, with a virtually complete subsidence of the organic mental syndrome.

16. In 1961 DeWind (1466) reported a case of excess ingestion of D by a boy which was accompanied by a severe degree of osteosclerosis. A five-and-a-half year old boy had ingested large quantities of D for one year after a rickets diagnosis. Radiographs showed generalized increase in bone density and no evidence of rickets. D dosage was discontinued. One year later however, there was a spectacular decrease in overall bone density (1466). At this time there was no evidence of renal failure. The child died shortly thereafter. Autopsy showed extensive calcification in the coronary artery, lungs, gastric glands, adrenal glands and kidney. The bone marrow showed osteoporosis.

The author attributed the severe calcinosis and fatal renal failure to the presence of a constant steady stream of Ca from bone to bloodstream as a result of the prompt reversal of bone density when D therapy was discontinued. The author attributed the quantitative increase of bone mineral initially seen to the excessive ingestion of D. He concluded that this case demonstrated that as a result of ingestion of large quantities of D over a year by a child,

there was a reversible increase in bone density with severe calcinosis and renal failure.

17. In 1962 Nigrin et al. (4269) reported 11 cases of refractory hypophosphatemic rickets all of which had been treated with large doses of D. Histological examination showed patchy distribution of intratubular Ca deposits either in the form of obstructing casts or as small particles and dilatation of tubules. Calcification was found in all cases except one. The authors concluded that irreversible renal changes could result with large doses of D, despite constant surveillance.

18. In 1963 Cuthbertson (1257) reported the D content of the sera of 11 children with idiopathic hypercalcemia as compared to plasma samples from healthy children. The results showed that the D levels for patients with the disease, either in mild or severe form, did not differ from each other or from those of the controls.

19. In 1964 Black (0589) reviewed the relationship of D to idiopathic hypercalcemia and compared the clinical pathology resulting from D overdosage to that of idiopathic hypercalcemia noting similarities. Three cases in which overdosage with D produced a clinical picture similar to that of severe hypercalcemia are summarized in Table 41. The significance of D activity in the serum is discussed. Some data from case histories are summarized in Table 42.

In conclusion the author noted that consideration of the facts concerning D toxicity is necessary in examining national policies concerned with the fortification of milks with vitamin D, as well as in the study of the current use and abuse of D by the medical profession and the general public.

20. In 1964, DeLuca (1320), reported on 12 infants between the ages of 7 and 30 months intoxicated by D overdosage. The cases are summarized in Table 43. The laboratory tests confirming the clinical signs of D intoxication are summarized in Tables 43, 44 and 45.

In 10 of the 12 cases discussed, a clearcut picture of renal tubular acidosis was established. The doses administered were from 48,000 IU to 200,000 IU daily with a total dosage between 1,200,000 and 10,000,000 IU. The authors considered the daily dose more significant with respect to effect on the clinical picture than the total dose. The authors noted the presence

Table 41

Cases of Hypercalcaemia Resulting from Large Doses of Vitamin D (0589)

Case*	1	2	3
Typical symptoms	+	+	+
Facies	?	+	+
Squint	0	+	0
Mental Retardation	+	?	?
Osteosclerosis	+	0	+
Systolic murmur	0	0	0
B. P.	175/120	?	145/95
Hypercalcaemia	+	+	+
Raised blood urea	+	0	+
Serum cholesterol	310 mg.%	200—216 mg.%	485 mg.%
Symptoms after giving Vitamin D	Yes	Yes	Yes
Total dose of vitamin D	2,000,000 I.U. "D"	40 mg. D_2 (1,600,000 I.U.)	6,800,000 I.U. D_3
Period of treatment with vitamin D (age in weeks or months)	6/12—20/12	6/52—11/12	4/12—10/12

*Case 1. Lang and Kiardt (31), Case 2. Koranyi (30), Case 3. Amann (1).

Table 42

Cases of Idiopathic Hypercalcaemia (Vitamin D Assays on Serum @589)

Authors	Vitamin D intake	Clinical type	Vitamin D assay results IU per ml.	Period without vitamin D
Lang & Elardt (31)	Excessive	Severe type* hypercalcaemia	4	2 months
			2.5	8 months
Fellers (16)	Normal	Severe type hypercalcaemia	10	0
			19	18 months
Fellers (16)	Normal	Severe type hypercalcaemia	20	0
			28	16 months
			<2	26 months
Smith, Blizzard, and Harrison (47)	1500 units daily, then 4,400 units daily for 1 month before admission	Severe type hypercalcaemia	23	0
			5	6 months
			2	21 months
Schmid, Just & Stalder (45)	300,000 IU 2 months before admission & 300,000 IU just before admission	Mild type hypercalcaemia	1.75	Not stated
Hovels & Stephan (25)	Not stated	Intermediate hypercalcaemia	3.6	Not stated
Thomas Morgan Connor, Haddock, Bills & Howard (51)	Not stated	Mild** (2 cases)	"Normal"	Not stated
Cuthbertson (11)	1350	Mild	2.9	0
	2000	Mild	3.0	3 days
	1000	Mild	1.5	6 days
Cuthbertson (14)	Not stated	Mild	2.0	7 days
	2500	Mild	2.1	3 1/2 weeks
	2000-3000	Mild	1.4	
	1500	Severe	2.0	0
			2.0	12 days
	1000	Severe	1.7	3 weeks
	3000	Severe	2.0	3 1/2 weeks
	1700	Severe	4.0	6 1/2 months

*Probably a case of vitamin D overdosage

**Serum obtained from 2 cases in Britain (Mitchell—personal communication).

Table 43 (1320)

Observation		Purpose	Doses of Vit. D administered		Symptoms which led patient to our observation											
M Name, sex, age	Date of admission to hospital	of prescription	Total mg	per day mg	Anorexia	Stypsis	Vomiting	Weight loss	Degree of anemia	Polyuria	Polydipsia	Hyperthermia	Convulsions	Toxic state (skin dehydration)	Asthenia	Personality changes
1) Z.E. F. 13 mo.	1-9-1959	Tonic	120	1.2	+	+	+	+	++	+	+		+			
2) B.M. F. 10 mo.	1-7-1959	Tonic	120	4	+	+		+	++	+	+	+		+	+	
3) C.D. F, 1 yr., 1 mo.	20-8-1960	Antirachitogenic (prevention of rickets)	250	1.5	+	+	+	+	+	+	+	+		+	+	Sleepiness
4) P.B. M, 9 mo.	15-10-1960	Prevention of rachitogenic (rickets) tetany	30	2.5	+	+		+	++	+	+		+	+	+	Instability
5) Z.P. M, 2 yr.	16-7-1961	Prevention of rachitogenic (rickets) tetany	105	2	+	+		+	++	+	+			+	+	Instability
6) C.G. M, 10 mo.	1-3-1963	Antirachitogenic prophylaxis (prevention of rickets)	150	1.6	+	+	+	+	++	+	+			+	+	Instability
7) M.G. F, 1 yr.	27-3-1963	Tonic	50	1.7	+	+		+	+	+	+	+		+	+	Restlessness
8) C.A.M. F, 7 mo.	17-4-1963	Tonic	50	1.6	+	+	+	+	+	+	+			+	+	Restlessness
9) N.A. F, 1 yr., 1 mo.	5-6-1963	Tonic	25	1.5	+	+		+	++	+	+			+	+	Restlessness and instability

Table 43 (cont'd)

	Observation		Purpose	Doses of Vit. D administered		Symptoms which led patient to our observation										Personality changes
	M Name, sex, age	Date of admission to hospital	of prescription	total mg	per day mg	Anorexia	Stypsis	Vomiting	Weight loss	Degree of anemia	Polyuria	Polydipsia	Hyperthermia	Convulsions	Toxic state (skin dehydration)	Asthenia	
10)	C.N. F, 1 yr. 1 mo.	1-7-1963	Tonic, Antirachitogenic prophylaxis (prevention of rickets)	60	1.5	+	+	+	+	++	+	+	+		+	+	Restlessness
11)	C.M. F, 1 yr.	10-7-1963	Tonic	60	1.5	+	+	+	+	++	+	+	+		+		Restlessness
12)	M.L. M, 2 yr. 4 mo.	26-7-1963	Tonic	76.5 + durab.	1.7	+	+	+	+	++	+	+	+				Apathy

Table 43 (cont'd)

	Case history data	Embryological classification of Toni-Gobessi (Genoa media)	Arterial pressure	E.C.G.	E.E.G.	Pitressin test	Alkalinization test	Radiological signs	Therapy	Evolution
1) Cystitis; convulsive fits	Maternal feeding		105/75		+	Neg.				Favorable
2) Cystitis	Maternal feeding		105/75	+		Neg.				Favorable
3) Cystitis	Parents related by blood, maternal feeding	Pachisomia	125/75			Neg.		+	Alkalizing rehydration agent	Favorable Favorable
4) Convulsive crises	Maternal feeding	Typosomia	95/55		+	Neg.		-	Alkalizing rehydration agent	Favorable
5) Convulsive crises	Maternal feeding	Microsomia	80/55	+	+	Neg.		+	Alkalizing rehydration agent	Favorable
6) Cystitis	Maternal feeding	Hyposomia	90/55	+	+	Neg.		+	Alkalizing rehydration agent	Favorable
7) Personality changes	Maternal feeding	Nanism, leptosomia	80/50	+	+	Neg.	Pathological result	-	Alkalizing rehydration agent + Dexamethasone treatment (T)	Favorable

Table 43 (cont'd)

	Case history data	Embryological classification of Toni-Gobessi (Genoa media)	Arterial pressure	E.C.G.	E.E.G.	Pitressin test	Alkalinization test	Radiological signs	Therapy	Evolution
8) Cystitis	Maternal feeding	Typosomia	85/55	+	+	Neg.	Pathological result	+	Alkalizing rehydration agent + Desametazone treatment (?)	Favorable
9) Cystitis	Maternal feeding	Macroleptosomia	95/60	+	+	Neg.		-	Alkalizing rehydration agent	Favorable
10) Toxicoses	Maternal feeding	Hypersomia	105/70	+	-	Neg.	Pathological result	-	Alkalizing rehydration agent + Desametazone treatment (?)	Favorable
11) Personality changes	Maternal feeding	Typosomia	90/65	+	-	Neg.		+	Alkalizing rehydration agent + Desametazone treatment (?)	Favorable
12) Personality changes	Maternal feeding	Typosomia	85/50	+	-	Neg.	Within normal limits	-	Alkalizing rehydration agent	Favorable

Table 44

Case No.	Ca	P	K	Na	Cl	Alkaline Reserves	Alkaline phosphatases UKA	Azotemia	Cholesterolemia	Glycemia	Electrophoresis A	α_1	α_2	β	γ	A/G	Total Proteins
1)	13.4	4.5	—	—	—	—	—	32	—	—	—	—	—	—	—	—	—
2)	17	4.9	13.5	340	331	50	13	25	130	1	—	—	—	—	—	—	6.5
3)	11.8	4.5	—	—	510	—	6	28	—	—	—	—	—	—	—	—	—
4)	11.9	5.1	—	—	—	38	6	26	—	—	—	—	—	—	—	—	—
5)	11.5	4.8	—	—	—	38	8	24	—	—	—	—	—	—	—	—	—
6)	10.7	4.2	—	—	—	41	3	30	—	—	—	—	—	—	—	—	—
7)	12.15	3.2	14.5	—	655	33	4	26	235	0.85	46	7	19	15	14	0.85	5.80
8)	10.85	4.7	15	305	381	35	8	32	270	0.90	46	7	17	12	19	0.85	6.90
9)	11.15	5.3	—	—	—	42	15	23	—	—	—	—	—	—	—	—	—
10)	13.05	3.2	17	285	549	30	6	26	196	1.15	47	7	17	16	13	0.88	7.20
11)	12.4	4.5	—	—	—	41	7	24	220	—	46	7	19	15	13	0.85	7.90
12)	10.4	2.8	—	—	—	43	7	26	148	—	50	5	12	12	21	1	6.85

Blood Chemical Data Referring to Each Case Are Reported

Table 45

Case No.	Diuresis cc/24 hr.	Urine density 24 hour	pH of urine 24 hour	Albuminuria 24 hour	Urine sediment 24 hour	Ca urine 24 h	P urine 24 h	Na urine 24 h	K urine 24 h	Cl urine 24 h
1		1014	6			+++				
2		1008	8	—	++	162				2600
3	1300*	1006	7	+	+	250				
4	1300*	1004	8	—	—					
5	1700	1006	8	—	—	231				
6	1900	1004	8	—	—	380	340	2710	610	
7	1950	1004	8	—	—	368	1310	1820	810	1800
8	1000*	1011	8	—	+	240	1630	1270	680	1914
9		1006	7	—	—	261				
10	1100*	1007	8	+	+	215	1267	1590	390	2810
11.	1750	1005	8	+	+	321	614	700	342	855
12	1200		7	—	—	190				

*The data listed in the table are partial figures, due to the fact that a complete collection of urine was not possible in view of the age of the patients.

of psychological disturbances in all the cases: two had convulsions and ten exhibited character disturbances.

One fact pointed out by the authors was that 10 of the 12 cases occurred during the spring and summer, when they postulated that there is a greater endogenous ergosterol and calciferol production owing to increased UV irradiation, thus lessening the requirement for exogenous D.

21. In 1965 Coleman (1083) reported having found electrocardiographic changes in patients with infantile hypercalcemia. He considered these changes as indicative of left ventricular myocardial damage. He produced experimental lesions of the aortic wall and of mural endocardium by giving high D_2 and D_3 doses to young rabbits (for details see p. 77).

The author's conclusions were:

a. The possibility existed that congenital endocardial fibroelastosis and the myocardial lesion of fibrocystic disease of the pancreas were related to D.

b. The persistence of infantile hypercalcemia and the possibility of residual cardiovascular lesions were disquieting.

c. Responsible inquiry should be made into the nature of such residual effects and their relative importance.

22. In 1966, Beuren et al. (0526) presented a clinical report without laboratory data concerning 37 children in Germany with supravalvular aortic stenosis (SAS), all of whom also had pulmonary artery stenoses. In this latter group were 22 with the typical facies of hypercalcemia and mental retardation. These patients had received repeated massive D doses, sometimes as high as 600,000 IU. Eleven more patients had a generalized hypoplasia of the aortic and pulmonary arteries. The authors pointed out that the number of patients suffering from hypercalcemia or D hypercalcemic cardiovascular disease was relatively high in Germany and greater than in countries where more physiological prevention of rickets was practised.

In 1967, A Commission of the German Pediatric Society (5153) studied this and other reports and concluded the following:

a. SAS syndrome including both the described external and vascular changes did exist.

b. There appeared to be a connection between this syndrome and the severe form of idiopathic hypercalcemia.

c. There may be a constitutional hypersensitivity towards D in some children and their mothers.

d. SAS has been found in some offspring of pregnant animals administered toxic D doses.

e. It had not been confirmed (in 1967) that a relationship existed between this syndrome and massive D doses for prevention of rickets.

f. In those individuals having a hypersensitivity to D, any administration of this vitamin could contribute to the development of the syndrome during pregnancy and after birth.

g. It would be desirable to have a test for detecting individual hypersensitivity to D.

h. Continuous therapeutic (500 to 1000 IU) doses of D are preferable to single massive doses for rickets prevention in infants.

i. The margin between a therapeutic and toxic dose of D is only five-fold in rats.

j. No opinion was arrived at concerning addition of D to milk for rickets prevention.

23. In 1966 Taussig (5703) reviewed idiopathic hypercalcemia of infants, drawing attention to aspects that had been mentioned but not emphasized by predecessors including Bongiovanni et al. (0656). Cardiac murmur without apparent pathology had been part of the syndrome: pathology was now identified as SAS. Taussig suggested that this might reflect a congenital malformation, associated with D-hypersensitivity. The author concluded with a plea to physicians to avoid giving amounts of D that were unnecessary and might be harmful.

24. In 1968 Anderson et al. (0090) reported the cases of two patients suffering from depressive symptoms resulting from D intoxication. In one case a woman (65 years old) with a previous history of manic-depressive psychosis was given 150,000 IU of calciferol daily. Before D intoxication was diagnosed, she became depressive, developed a toxic confusional condition with hypernatremia and hypokalemia. After D was stopped, there was a dramatic improvement: plasma calcium fell and she rapidly regained consciousness. Eleven days later she appeared normal and was not depressed.

In the second case a man (73 years old) was put in a psychiatric hospital

for manic-depressive psychosis/depressive reaction. He had been treated for two years for osteomalacia with 600 mg calcium gluconate twice daily and 100,000 IU calciferol daily. There was rapid improvement once the Ca and D were withdrawn with a disappearance of his depression and hypochondriacal delusions.

The authors considered that in their two patients with a known history of manic-depressive psychosis, the return of their depressive symptoms shortly before the appearance of physical symptoms of D intoxication, together with the rapid improvement in their depression after treatment, suggested that D intoxication may have been important in precipitating and maintaining the depression to which they were both predisposed.

25. In 1968 Paunier et al. (4473) reported the cases of 14 patients with D refractory rickets treated with high D dosages for long periods. In order to regulate the vitamin dosage, the patients and their parents were taught to recognize the early clinical symptoms of hypercalcemia. The initial oral dose of D was 25,000 to 50,000 IU/day. The dosage used to produce optimal healing was between 50,000 and 250,000 IU/day. The incidence of hypercalcemia episodes and the degrees of hypercalcemia are shown in Table 46.

Each of nine patients had a single episode of D intoxication. Upon diagnosis, D therapy was either reduced or temporarily stopped. In one case it took ten days for the serum Ca to return to normal· another patient, the only one in the series who had a history of previous severe and protracted D intoxication, had impaired renal function, chronic pyelonephritis. Calcium deposits were seen with light microscopy.

Each of the 13 other patients in the series had normal renal function. This led the authors to conclude that if carefully controlled, long-term D therapy can be relatively safe. They pointed out that an increase of the serum Ca level above the upper limit of normal when frequently and accurately determined, provides a reliable signal of D toxicity.

26. In 1970 Gabriel et al. (2009) reported the case of a woman with anemia and renal failure due to D toxicity. The main symptoms were a reduction in renal function and an 18-month history of an unexplainable variable anemia, 7-10 g/100 ml. Plasma Ca was 14.5 mg/100 ml, P_i 3.6 mg/100 ml with a urinary Ca of 325 mg/day.

Case	Sex	History No.	Type of refractory rickets*	Chronological age (years)	Height age+ (years)	Dose of vitamin D_2 (I.U./day)
1	M	183471	Aminoaciduria	16	12 1/2	50,000-70,000
2	F	247040	Aminoaciduria	15	11 1/2	75,000-100,000
3	F	244412	Simple	16	12 1/2	50,000-175,000 (stopped at age 14 yr.)
4	F	253823	Simple	15	11 1/2	100,000-125,000
5	M	198426	Simple	14 1/2	11	75,000-100,000
6	M	271139	Simple	11 1/2	9 1/2	75,000-100,000
7	F	436138	Simple	13 1/2	10 1/2	100,000
8	M	397715	Simple	11	8	100,000-125,000
9	M	302011	Simple	11 1/2	6 1/2	100,000-125,000
10	F	266997	Simple	12 1/2	11 1/2	75,000-100,000
11	F	317480	Simple	12 1/2	7 1/2	150,000
12	M	395252	Aminoaciduria	7 1/2	6	25,000-50,000
13	F	398432	Simple	7	5	25,000-50,000
14	F	523188	Simple	14	5	250,000
Normal values						

*Abbreviations for types of refractory rickets: simple = vitamin D refractory rickets of the simple type[6, 7]; aminoaciduria = vitamin

+From data of Stuart, H. C., et. al., Department of Maternal and Child Health, Harvard School of Public, Boston, MA

Table 46 (cont'd)

Case	Duration of therapy (years)	Serum Ca‡ (mg/100 ml)	Serum P‡ (mg/100 ml)	Serum alkaline phosphatase‡ (K.A. units)	Hypercalcemic episodes
1	14	9.3	2.2	35	0
2	12 1/2	9.0	3.9	12	Age 6 yr.: Ca 16.6 mg/100 ml had returned to normal in 4 mo.
3	12	8.9	1.2	11	Age 5 yr.: A single Ca of 12.8 mg/100 ml
4	11 1/2	9.6	1.2	15	Age 6 yr.: Ca 15.7 mg/100 ml had returned to normal in 6 mo.
5	10 1/2	9.4	2.5	22	Age 12 yr.: Ca 14.1 mg/100 ml had returned to normal in 4 mo.
6	9 1/2	9.0	2.9	25	0
7	9 1/2	9.4	2.3	18	Age 11 yr.: a single Ca of 11.2 mg/100 ml
8	9	9.8	2.7	26	0
9	9	10.0	3.0	20	Age 6 yr.: a single Ca of 11.1 mg/100 ml
10	8 1/2	9.9	2.5	17	Age 6 yr.: Ca 14.3 mg/100 ml had returned to normal in 1 mo.
11	8	9.6	2.1	16	0
12	6 1/2	9.0	5.2	19	Age 14 mo.: Ca 15.8 mg/100 ml had returned to normal in 10 days
13	6	9.7	2.6	14	Age 1 yr.: severe, protracted hypercalcemia (see text)
14	1 1/2	9.3 9.0–10.5	1.9 3.0–5.5	19 10–20	0

‡ Mean values on treatment.

D dependent rickets with aminoaciduria, [7, 27]

The patient's history showed that for the previous 7-8 years she had regularly taken 100,000 IU of D daily, initially prescribed for fingernail-splitting. The D was withdrawn. The patient however, remained ill and developed a mental depression. After one month of treatment, when the Ca levels fell to normal, good health returned. Both the severe renal functional impairment, (function was one quarter that of normal) and the hemoglobin concentration improved. The hematological improvement followed withdrawal of D and occurred without any hematinics. The authors postulated that hypervitaminosis D was a direct or indirect cause of the anemia, possibly acting by interfering with the renal production of some substance affecting erythropoiesis or red cell survival.

Tissue obtained from the kidney by needle biopsy did not show any Ca deposits. There was no evidence of bone sclerosis and the skeleton was radiologically normal. The authors pointed out that D intoxication could also be manifested as a neuropsychiatric problem.

27. In 1971 Lumb et al. (3607) reported the case of a 14-year-old girl with chronic pyelonephritis and azotemic rickets who was given 18,000 IU of ergocalciferol daily. After four months as an outpatient, her serum Ca was 15.2 mg/100 ml and serum D activity was 72 IU/ml. The authors considered hypercalcemia at the dosage level unusual. Therefore they postulated that the patient was incorrectly given six calciferol tablets daily, each containing 50,000 IU rather than 3,000 IU as had been prescribed.

III. Long-Term Studies

A. Mice

Robertson et al. (4854) studied the effect of a moderate overdose of D on the growth rate and longevity of the white mouse. Two groups each of 36 male white mice were fed on a mixed diet. One group was additionally given 50 rat-units of D dissolved in 0.05 ml of olive oil daily. The other group which acted as normal controls received only 0.05 ml of olive oil daily. The results are summarized in Tables 47 and 48.

It was observed that:

a. From 90 weeks of age to death, there was no difference in the weights of the two groups of animals.

Table 47

Group M. Controls (4854)

Number of Animals	Age in weeks	Mean weight in Grammes	Probable error in Grammes
25	100	30.14	.35
24	102	30.38	.31
23	104	30.50	.46
22	106	30.66	.42
22	108	30.23	.39
22	110	29.86	.42
22	112	29.30	.36
22	114	28.57	.42
19	116	28.97	.53
18	118	29.28	.56
17	120	29.12	.52
17	122	28.94	.42
15	124	27.63	.57
13	126	27.88	.55
11	128	27.64	.68
11	130	26.86	.73
9	132	27.89	.70
8	134	28.62	.83
7	136	27.64	.84
6	138	25.83	.80
4	140	25.87	—
3	142	25.17	—
3	144	24.50	—
2	146	28.25	—
2	148	28.75	—
2	150	27.55	—
2	152	25.75	—
1	154	30.50	—
1	156	30.00	—
1	158	30.50	—
1	160	30.00	—
1	162	30.50	—
1	164	31.00	—
1	166	30.00	—
1	168	29.50	—
1	170	28.50	—
1	172	28.50	—

Table 47 (cont'd)

Group N. vitamin D

Number of Animals	Age in weeks	Mean weight in Grammes	Probable error in Grammes
26	100	30.50	.54
24	102	31.02	.47
24	104	30.71	.55
23	106	29.61	.64
21	108	30.07	.56
21	110	29.55	.66
18	112	28.69	.62
15	114	29.97	.65
15	116	29.87	.55
14	118	28.93	.70
14	120	28.89	.63
13	122	28.92	.59
13	124	28.38	.55
13	126	27.77	.59
13	128	28.46	.67
13	130	28.08	.62
13	132	27.23	.56
10	134	27.70	.59
9	136	27.72	.52
8	138	26.87	.66
6	140	28.25	1.08
5	142	28.40	1.01
5	144	27.30	---
4	146	26.62	---
4	148	26.12	---
4	150	25.12	---
2	152	24.50	---
2	154	27.75	---
2	156	26.25	---
2	158	22.00	---
2	160	21.00	---
1	162	24.00	---

Table 48

MORTALITY STATISTICS

(Accidental deaths excluded) (4854)

At Age in Days	Percentage of Survivors Group M. Control	Group N. vitamin D
200	100	100
250	100	100
300	100	100
350	100	100
400	100	97.1
450	100	97.1
500	100	94.3
550	100	85.7
600	94.3	85.7
650	82.9	82.9
700	71.4	74.3
750	62.9	62.9
800	60.0	42.9
850	48.6	37.1
900	34.3	28.6
950	20.0	28.6
1,000	11.4	14.3
1,050	5.7	11.4
1,100	5.7	5.7
1,150	2.9	0.0
1,200	0.0	

Mean Duration of Life
Group M 825±17 days
Group N 806±22 "

Mean Duration of Life of Mice still alive at 750 days
Group M 924±14 days
Group N 920±17 "

Mean Duration of Life of Mice dying before 750 days
Group M 650± 9 days
Group N 612±21 days

Difference between M and N 46±23 days

b. The life span of the animals receiving D was slightly less than that of the controls.

B. Rats

In 1929, Bills and Wirick (0566) studied the effects of administering activated ergosterol to rats from infancy to old age. The activated ergosterol solution used in most of these experiments was standardized to contain 100 times the D content of average cod liver oil.

In the long-term feeding experiments, activated ergosterol was administered to a total of 231 young rats (including controls) in doses 100, 1000, 4000 and 40,000 times greater than the minimum antirachitic level.

It was observed that with respect to the parameters of general appearance, growth, reproduction, and resistance to respiratory infections, the dose 100 times greater, showed no effect; the 1000 times overdosage was just perceptibly harmful; 4000 times overdosage was definitely injurious and 40,000 times overdosage was strongly toxic.

The authors concluded that the effects of overdosage with activated ergosterol became evident when 4000 times the minimm antirachitic dose was administered over an extended period.

C. Humans

In 1958 Gillman (2101) gave reasons for suspecting that human arteries were most susceptible to metabolic injuries during the first two years of life, regarding such injuries as precursors of adult atherosclerosis.

IV. Special Studies

A. Various Species (Comparative)

In 1931 Taylor et al. (5716) studied the relative effects of excessive doses of irradiated ergosterol in different species.

a. Cats were found to respond less readily than dogs to poisoning with irradiated ergosterol.

b. Rats were found to be even more resistant. Calculated on a weight basis, a 300 g rat was resistant to a dose of irradiated ergosterol at least 100 times that producing a definite response in dogs.

c. Mice were found to be somewhat less resistant than rats.

d. Rabbits were found to possess a fairly high resistance to irradiated ergosterol, surviving for 10 days on a daily dose at least 10,000 times the antirachitic rat dose. It was difficult to raise the serum calcium level of rabbits with irradiated ergosterol.

e. Guinea pigs were found to be from two to three times as resistant to the action of irradiated ergosterol as rabbits.

f. From experiments with chickens, it was concluded that when on a diet in which the Ca-P ratio is approximately normal, fowl were practically immune to the toxic effects of irradiated ergosterol.

The authors concluded from the comparison of different species, that for those species studied -- rats, chickens, dogs and humans -- the antirachitic dose was of the same order of magnitude: however, the tolerance to excessive doses varied enormously. This is summarized in Table 49, 50. The authors noted that there was clinical evidence that humans may share with dogs a high susceptibility to irradiated ergosterol.

Table 49

Curative Doses of Irradiated Ergosterol, and Cod Liver Oil in Different Species (5716)

	Irradiated Ergosterol	Cod Liver Oil
Species	Rat units per 100 grams animal per day	Rat units per 100 grams animal per day
Rats	1.3	1.7
Chickens ...	1-2	1-2
Dogs	?	1-2
Infants	Dose prescribed varies from 2-10	1-2

The values in both the foregoing tables are of necessity only approximate.

B. Mice

1. In 1934 Agduhr (0040) continued earlier investigations with white mice in which he found that irradiated ergosterol increased the fecundity of the animals and that normal sexual activity improved the resistance of the mice to the toxic effects of large amounts of ergosterol.

This follow-up series was carried out as follows with a total of 97 white mice (52 males and 45 females) all fed a basal diet of whole milk, bread and oats:

Table 50

Lethal Doses of Irradiated Ergosterol (5716)

Species	Irradiated Ergosterol Dose in rat units* per 100 grams of animal Total	Daily
Rats	3,000,000-4,000,000	40,000
Mice (Young)	150,000- 200,000	50,000-75,000**
Chickens (5 mos. old)	600,000- 800,000	25,000
	causes no appreciable effect	
Guinea-pigs	150,000- 200,000	8,000-10,000
Rabbits	40,000- 60,000	3,000- 5,000
Cats	8,000- 10,000	600- 1,200
Dogs	6,000- 8,000	400- 1,000
Infants	?	?

* A standard rat unit is taken as the minimal daily amount (75 mg) of a good average cod liver oil which will induce healing in a 60 g rachitic rat in 10 days. The potency of a preparation of irradiated ergosterol is computed upon this basis. Thus irradiated ergosterol having a potency of 100 or 250 D has weight for weight 100 or 250 times respectively the antirachitic value of cod liver oil.

** The toxicity of irradiated ergosterol depends to a large extent upon the size of the daily dose as well as upon the total amount given. In the experiment which we have undertaken with this species a relatively large daily dose was given. The survival time was consequently probably shorter than if smaller doses had been given. The daily lethal dose is probably considerably less than the figure given here and the total lethal dose may be much greater.

Series A and B: Each mouse received 200 μg daily of irradiated ergosterol p.o.

Series C: Each mouse received 100 μg dailyof irradiated ergosterol p.o.

Series D were controls.

The series A and C mice were prevented from normal sexual intercourse, whereas the series B and D mice were kept together in male-female pairs. The experiments and their results are summarized in Table 51.

Some of the observations were:

a. In the series A mice (17 males and 13 females), the mortality was much higher for the males than for the females (64.7 vs. 7.7% respectively). The deaths were considered to be the result of ergosterol toxicity.

b. In the series C mice (21 males and 18 females), the mortality was again much higher for the males than the females (47.6 vs. 11.1% respectively). Again death was attributed to lesions caused by the ergosterol.

c. In the series B mice (14 males and 14 females), only one male and no females died during the experiment.

Some of the conclusions drawn were:

a. Male mice were much more susceptible to irradiated ergosterol overdosing than females.

b. The larger dose (200 μg) of irradiated ergosterol to the paired mice increased the number of pregnancies despite the lesions which were caused by the ergosterol.

The author noted that in a previous paper published in 1932 he had reported an accelerated weight increase in the pituitary gland of ergosterol-treated females, but not in similarly treated males. He also reported an effect on the thyroid and parathyroid glands. From this he concluded that the increased mortality in the ergosterol-dosed males as compared to the females was the result of a more severe endocrine disturbance which lowered their resistance to the toxicity of the irradiated ergosterol.

2. In 1942 Francioni (1885) studied the blastomogenic effect of D_2. Three experiments were carried out:

a. On alternate days for 6 to 11 months, 15 mice (specifications not given) were painted with D_2. Three different types of neoplasms (neoplastic leukemic lymphadenoma, mammary adenoma, and subcutaneous spindle-cell sarcoma) were noted in 20% of the mice at points distant from the treatment site.

b. Another 15 mice received a dorsal s.c. injection of D_2. After 11 months in 6.6% of the animals, an Ehrlich-type mammary adenocarcinoma was found in a site distant from the injection site.

Table 51

Number of the group	Ergosterol, irradiated with ultra-violet light: µg per mouse per day	Rats-Units of D-Vitamin	Number of mice ♂	Number of mice ♀	Age at the beginning of the experiment: Days	Length of the experiment time: Days	Bodyweights gr. at the start of the experiment	Bodyweights gr. at the end of the experiment	Bodyweights gr. at the middle of the experiment	Basal-diet: Bread, unskimmed milk and oats	Beginning of the experiment
233	200	2000	4		32	286	13,2	26	24	1	16, 11, 31
234	200	2000	3		36	152	10,9	18,4	17,2	1	16, 11, 31
235	200	2000		5	36	265	11	22,4	19,6	1	16, 11, 31
236	200	2000	4		32	258	6,5	23,2	20,2	1	23, 11, 31
243	200	2000	6		30	290	10,1	26,7	25,4	1	29, 12, 31
244	200	2000		4	35	290	12,1	21,9	20,5	1	12, 1, 32
245	200	2000		4	32	272	11,8	24,7	22,2	1	19, 1, 32
237A	200	2000	2	2	32	310	9,7	26,7	23,9	1	23, 11, 31
237B	200	2000	2	2	32	316	10,6	31,3	27,1	1	23, 11, 31
238A	200	2000	2	2	32	270	11,1	26,5	23,2	1	7, 12, 31
238B	200	2000	2	2	32	270	10,1	27,8	26	1	7, 12, 31
239A	200	2000	2	2	36	280	11,2	27	25,5	1	7, 12, 31
239B	200	2000	2	2	36	280	11,4	28,7	26,1	1	7, 12, 31
249	200	2000	2	2	36	272	10,6	28,6	26,2	1	2, 2, 32
228	100	1000	6	2	40	270	15,2	25,5	23,3	1	30, 9, 31
231	100	1000		4	30	283	11,2	22,5	20,6	1	25, 10, 31
232	100	1000	5		32	286	14,2	25,8	23,6	1	25, 10, 31
246	100	1000	4		34	278	12,9	32,3	29,6	1	25, 1, 32
247	100	1000		4	36	272	11,9	27,7	22,9	1	25, 1, 32
248	100	1000	6		30	272	12,5	25,5	24,4	1	25, 1, 32
251A	100	1000		4	36	272	10,2	22,2	21,8	1	2, 2, 32
251B	100	1000		4	36	272	8,8	26,1	27,1	1	2, 2, 32
Controls on the basal diet only:											
320	—	—	2	2	30	320	8,1	30,4	25,9	1	8, 3, 33
324	—	—	2	2	30	314	11	26,1	25,7	1	15, 4, 33
328	—	—	2	2	32	303	8,9	25,5	24,3	1	22, 4, 33
331	—	—	2	2	34	303	10,9	28,6	26,2	1	23, 4, 33
Controls on the basal diet and on the solvent:											
144	—	—	2	2	30	220	12,6	26,6	25,6	1	2, 1, 31
159	—	—	2	2	31	244	14,2	25,6	25,2	1	2, 1, 31
144b, 159b	—	—	8		30	240	11,3	27,8	26,4	1	10, 1, 32

Table 51

Animals diet: Number of the mice ♂	♀	Average days of the experiment ♂	♀	Average number of the gravidities per female	The mice were kept: Male and female together in pairs in different rooms of the same cage	Separated from each other in different rooms of the same cage	Together in the same cage	Quantity solvent (refined soy-oil) received per day per mouse cc.	Percentage of deaths: Males	Females	Series and its total number of mice ♂	♀
2		106				1		1/25				
3		128				1		1/25				
	1		13			1		1/25			A	
3		167				1		1/25	64,7	7,7	17	13
3		169					1	1/25				
	--						1	1/25				
	—					1		1/25				
--	--			8	1			1/25				
--	--			11	1			1/25				
1	--	124		6	1			1/25			B	
	--			7,5	1			1/25	7,14	0	14	14
	--			8,5	1			1/25				
	—			9	1			1/25				
	--			11	1			1/25				
2		11				1		1/50				
	1		161			1		1/50				
3		132				1		1/50			C	
2		176				1		1/50	47,6	11,1	21	18
	1		212			1		1/50				
3		225				1		1/50				
	--					1		1/50				
	--					1		1/50				
—	—	—	—	6,5	1							
--	--	--	--	6,5	1							
--	--	--	--	4	1							
--	--	--	--	4	1						D	
											20	12
--	--	--	--	4	1			1/50				
--	--	--	--	6,5	1			1/50				
—	--	--	--	—		1		1/50				

c. There was no tumor formation in 10 rats 26 months after a dorsal s.c. injection of D_2.

d. In 30 controls after 18 months, an Ehrlich-type mammary carcinoma was found in 3.3%.

3. In 1957, Hieger (2529), tested the carcinogenicity of cholesterol. Previous experiments by the same researcher (1947, 1949 and 1954) had produced evidence of cholesterol's carcinogenicity.

Purified cholesterol was tested on 224 mice (Stock or C57 strains) divided into three groups. The results are summarized in Table 52. It was observed that the sarcomas which developed in three of the mice kept in the carcinogen-free room had an unusually short latency period for cholesterol-induced tumors (8.5, 9 and 10 mos.). Another series of experiments was performed to show the results of injecting the same material in four separate groups of mice of the same strain. The results are summarized in Table 53. The author concluded from the above results that:

a. About 10% surviving a minimum of one year developed sarcoma at the injection site.

b. Purification did not alter cholesterol's carcinogenicity.

Compounds related to cholesterol were also tested. The results are shown in Table 54. None of the sterols tested showed appreciable carcinogenic activity. When the role of the solvent was tested it was found that:

a. Only oily solutions of cholesterol showed carcinogenic activity.

b. Cholesterol suspended in a 4% gelatin gel was not found to be carcinogenic.

c. Control tests with 700 mice treated with heated olive oil or lead, produced two sarcomas.

The author concluded that the role of the oil in cholesterol carcinogenesis was to ensure solution.

4. In 1974 Tashjian et al. (5699) studied the transplantable bone sarcoma of mice, $HSDM_1$. This tumor produces a bone-resorption factor that, in culture, was found to enter the medium and to cause bone resorption in co-cultured normal fragments. The factor was isolated, part-purified, and bioassayed. Its activity in vitro was found to be equivalent to those of PTH, prostaglandins E_1 and E_2, or 1,25-$(OH)_2$-D: and 10^{-7} to 10^{-9} M concentrations were required for half-maximum resorption. Higher concentrations were needed of 25-OH-D, D itself, PGA, PGB, or cAMP. The hypercalcemia was transiently eliminated in vivo by indomethacin, a PGE_2 inhibitor.

Table 52

SARCOMA INDUCTION IN STOCK MICE BY HIGHLY PURIFIED CHOLESTEROL (SCHWENK PROCESS) IN OLIVE OIL (2529)

expt.	location	survival rate (months) at start	6	9	12	15	18	21	24	27	no. of sarcomas	latent period (months)	% incidence of sarcomas per 100 mice calculated on no. of mice at start	at minimal latent period
1	'non-carcinogen room'	109	107	102	(all mice killed: epidemic)						3	8 1/2, 9, 10	——	——
2	'carcinogen room'*	115	90	84	66	33	22	10	—	—	11	9 1/2, 11, 11, 16, 17, 17, 18, 20, 22, 22, 23	9-5	14
For comparison: a test + on stock mice in the 'carcinogen room' using commercial cholesterol in olive oil														
3	'carcinogen room'	100	—	—	75	64	45	32	10	2	8	11, 12, 16, 16, 17, 20, 20, 24	8	10 7

*For a detailed description of this term see Hieger & Orr (1954).
+The figures for this experiment have been reported previously (Hieger & Orr 1954).

Table 53

SARCOMA INDUCTION IN MICE BY INJECTION OF COMMERCIAL CHOLESTEROL IN OLIVE OIL (2529)

expt.	mouse strain	location	no. of mice at start	no. of mice surviving (months) 6	9	12	15	18	21	24	27	30	no. of sarcomas	sarcoma induction calculated per 100 mice: at start (%)	at 1 yr. (%)	latent period (months)
1*	C_{57}	non-carcinogen room	60	34	30	28	21	14	9	3	1	—	1	1-7	3-6	22
2	C_{57}	non-carcinogen room	30	10	10	10	9	7	7	5	(3 at 25 m.) epidemic (all killed)		1	3-3	10	23
3	C_{57}	non-carcinogen room	39	28	26	24	20	16	11+		epidemic (all killed)		3	7-7	13	12, 14, 18
4	C_{57}	carcinogen room	75	62	48	46	40	26	15	3	0	—	4	5-5	8-7	21, 22, 22, 23

*This experiment was reported (Experiment A, Table V) by Hieger & Orr (1954).

+Eleven mice were still alive in the 21st month of this experiment (no. 3). There can hardly be any doubt that but for the epidemic the percentage yield of sarcomas would have been still higher.

Table 54

TESTS FOR CARCINOGENIC ACTIVITY OF COMPOUNDS RELATED TO CHOLESTEROL.
INJECTIONS WERE MADE SUBCUTANEOUSLY, REPEATEDLY (2529)

expt.	compound	how administered	no. of mice at start, and surviving during expt.						no. of sarcomas
			at start	after 1 yr.	18m	24m	27m	30m	33m	
1	isocholesterol	15% solution in lard	10	9	5	4	0	--	—	0
2	isolumisterol	15% solution in lard	10	9	4	0	--	—	—	0
3	cholesterylene	(15%) saturated solution in lard	10	5	5	3	2	1	—	0
	cholesterylene not purified	10% solution in lard	10	9	7	2	1	0	—	0
4	ergosterol	15% solution in lard	10	10	3	0	--	--	—	0
5	Δ-^{4}cholestene	15% solution in lard	10	9	4	3	2	0	—	0
6	7-dehydrocholesterol	15% solution in lard	10	10	7	2	2	0	—	0
	7-dehydrocholesterol	15% solution in lard	10	9	4	0	--	--	—	0
7	epicholesterol+ allocholesterol	15% solution in lard	10	9	8	3	3	2	0	0
8	7-hydroxycholesterol (mixture of 7 (α) and 7 (β)	15% solution in lard	20	14	6	2	1	1	1	0
9	lathosterol	10% solution in olive oil	50	37	23	5	2	0	--	0
10	deoxycholic acid	1% suspension in olive oil + tristearin (5:1)	73	34	21	6	1	0	—	1*
11	Na-deoxycholate	1.2 g cholesterol + 60 mg deoxycholate + 12 g olive oil + tristearin	62	44	26	6	0	—	--	1+

*C_{57} mouse ♂, latent period; 21 months.

+C_{57} mouse ♂, latent period; 18 months.

Thus these authors found a hypercalcemia syndrome that was associated with malignant tumors and, apparently, with a prostaglandin, and they commented that the possible role of the prostaglandins in the etiology of the hypercalcemia should be studied. Relationships of PTH and D to this hypercalcemia syndrome were not discussed.

C. Rats

1. In 1956 Gillman and Gilbert (2099) used 300 rats (220 male, 80 female) aged 3-6 months to study the cardiovascular effects of recrystallized purified D_2 dissolved in destearinated peanut oil and given orally by dropper, in doses of 25,000 IU.

Five groups of rats were given 1, 2, 3, 4, or 5 daily doses. In each group rats were killed 2, 4, 8, 10, or 21 days later, and in the 5-dose group, additional rats were killed at 12-day intervals for 200 days. Tissues were preserved in neutral formalin, and were tested histochemically for Fe, Ca, lipids, and mucopolysaccharides.

A wide range of effects was observed in aortas, coronaries, myocardia, and valves:

a. In aortas, damage was first seen in the endothelia, media, or both, and further damage depended on where it started. Calcification was common.

b. In the coronaries, calcification was diffuse or local, and at worst involved the entire vessel wall: in some rats, damage was as described for the aortas, with or without subsequent calcification.

c. Myocardial findings similar to the coronary findings were most frequent in the septum, the base of the heart, below the epicardium, the left ventricle, the endocardium, and the subendocardium.

d. The bases of the aortic and pulmonary valves and the surfaces of the mitral and tricuspid valves were the main sites of valve damage, and calcification was uncommon.

See the paper itself for detailed descriptions of the lesions and their progress.

To interpret their findings the authors reviewed literature in several languages. In 1930 Duguid had attributed vitamin D-arteriosclerosis to hypercalcemia induced by overdoses of the vitamin. However, in 1939 Reed, Struck, and Steck had noted that high doses of D and high blood Ca and P levels could each occur without the other: several other authors had added that D could be toxic without raising serum Ca.

The authors concluded that despite these differences of data, the evidence that overdoses of D could calcify soft tissues was overwhelming, but was

subject to much individual variation. They believed that much but not all of the damage was secondary to D-induced damage in the kidneys, but reversibility of the vascular damage depended entirely on reversal of kidney damage.

In a long discussion on the possible relevance of rat findings to atherogenesis in man, the authors concluded that in detail their findings appeared to be relevant. However, in man they pointed to ethnic "fundamental differences in calcium and phosphorus metabolism determined by diet."

2. In 1960, Chiaone (1024) studied the histological changes in the ovaries of D deficient and D overdosed albino rats. (See p.222 and p.150 for related studies by Oniwa and Kudo respectively). Table 55 shows the histological changes in D deficient immature rats. The author concluded that D-deficiency resulted in atrophy of the genitals with accompanying reduction in sexual function.

In the experiment with D overdosage the weights of ovaries of rats given 1000 IU or 5000 IU of D were greater than those of controls. Uteri in the group given 10,000 IU showed a tendency to be atrophied. The estrogenic effect of D in women has been reported by Freedman (1920).

The author concluded that a small amount of D stimulated follicular growth causing uterine thickening and acceleration of sexual function. A large, long-term dose however, caused first a transient stimulus but eventually caused the genitals to atrophy and arrested sexual function completely.

3. In 1968, Ornoy et al. (4389) investigated the effect of hypervitaminosis D_2 on the mineral composition of rat fetuses, fetal bones and placentas and on the maternal serum levels of Ca and P. A total of 24 pregnant and 12 nonpregnant albino rats (180-220 g) were administered 4000, 20,000 or 40,000 IU D_2 in 1 ml olive oil solution by intragastric intubation. Twelve controls received only olive oil. The animals were divided into eight experimental groups. The experimental results are summarized in Tables 56, 57, 58 and 59.

The significant results were:

a. Animals which received 40,000 units showed a statistically significant decrease of fetal wet weight, ash weight, and Ca and P contents (see Table 57).

b. Significant alterations in the composition of fetal bone were produced by 40,000 units. The concentrations of both Mg and P were considerably higher than controls (Table 58).

c. Placental weight was reduced in the groups receiving 20,000 and 40,000 IU D_2 (Table 59).

Tabl

The Ovary of Non-Sterilized Immature Rats Under D-Deficiency (1024)

	No. of Experimental Animal	Sexual Cycle	Weight	Blood Vessel	Interstitium	Initial Follicles	Major Follicles N	Major Follicles R	Medial Follicles N	Medial Follicles R	Minor Follicles N	Minor Follicles R	Closed Follicles	Luteins
The re-	51	O	35.5	C	N	++	2	1	3.5	2.5	7.5	6	average	12.5
covery	52	P	38	C	N	++	1	2	4.5	3	4	5.5	not so many	11
examina-	53	D	34.5	N	N	++	0	0	5	5.5	7	5	average	13
tion	54	D	38	N	N	++	0	0	2.5	4	5.5	5	not so many	11
group	55	M	36	N	N	+	0	1	3	3.5	6	4.5	not so many	10.5
The vit-	56	D	18.5	C	dense	++	0	0	0.5	2.5	2.5	0	many	0
amin D	57	M	33.5	N	N	+	0	0	1.5	2	3	4.5	many	8.5
defici-	58	D	37	N	N	++	0.5	1	1.5	2.5	3.5	4	not so many	11.5
ency	59	D	33	C	dense	+	0	0	2	2.5	4	8	many	9
group	60	D	13.5	C	dense	+	0	0	0	2.5	3	8	many	0

N = normal R = retrograde C = congested

Table 56

The Effect of Hypervitaminosis D_2 on the Serum Level of Calcium and Phosphorus in Pregnant and Nonpregnant Rats (4389)

Treatment	Nonpregnant			Pregnant		
	No. of rats	Ca (mg%)	P (mg%)	No. of rats	Ca (mg%)	P (mg%)
1 ml olive oil	4	10.0 ± 0.3[a]	6.4 ± 0.3	5	9.6 ± 0.3	5.2 ± 0.4
4,000 IU vitamin D_2	4	10.0 ± 0.2	7.0 ± 0.5	4	9.4 ± 0.6	6.5 ± 0.4
20,000 IU vitamin D_2	4	14.5 ± 0.9	5.2 ± 0.1	4	9.3 ± 0.3	6.7 ± 0.5
40,000 IU vitamin D_2		12.5 ± 0.4	7.8 ± 0.8	7	10.2 ± 0.5	6.1 ± 0.5

[a]Mean ± SE.
Significant statistical differences are shown by square brackets.

Table 57

The Effect of Hypervitaminosis D_2 on Wet Weight, Ash Weight, Calcium and Phosphorus Content of Whole Fetuses (4389)

Treatment	No. of fetuses	Wet weight of fetuses (mg)	Ash weight (mg)		Ca (mg)		P (mg)	
			Total	per 100 mg wet tissue	Total	per 100 mg wet tissue	Total	per 100 mg tissue
1 ml olive oil	18	5,130 ± 86[a]	75.8 ± 4.1	1.50	11.7 ± 0.2	0.23	14.2 ± 0.5	0.29
4,000 IU	12	5,195 ± 77	80.3 ± 1.6	1.55	11.0 ± 0.2	0.21	14.6 ± 0.2	0.28
20,000 IU	12	5,088 ± 137	77.2 ± 1.9	1.52	11.3 ± 0.3	0.22	15.1 ± 0.2	0.29
40,000 IU	18	2,514 ± 58	33.6 ± 0.9	1.33	4.3 ± 0.3	0.17	6.4 ± 0.4	0.25

[a]Mean ± SE.
Significant statistical differences are shown by square brackets.

Tab.

The Effect of Hypervitaminosis D_2 on the Wet Weight, Ash Weight, Calcium, Phosphorus and Magnesium Content of Pooled Fetal Bones[a] (4389)

Treatment	No. of fetuses	Wet weight (mg)	Ash weight (mg)	Ash (% of wet weight)	Ca		P		Mg	
					Total (mg)	Ash (%)	Total (mg)	Ash (%)	Total (mg)	Ash (%)
1 ml olive oil	18	9.7 ± 1.0[b]	2.44 ± 0.22	25	0.64 ± 0.03	26	0.45 ± 0.03	18	0.070 ± 0.010	3
4,000 IU	12	8.8 ± 0.7	2.33 ± 0.05	28	0.59 ± 0.03	25	0.39 ± 0.01	16	0.076 ± 0.005	3
20,000 IU	12	9.3 ± 1.0	2.23 ± 0.05	24	0.66 ± 0.06	29	0.40 ± 0.03	19	0.080 ± 0.020	4
40,000 IU	24	6.0 ± 0.9	0.88 ± 0.11	15	0.22 ± 0.05	25	0.23 ± 0.03	26	0.090 ± 0.018	10

[a]Two femurs, tibias and fibulas from every fetus. [b]Mean ± SE.
Significant statistical differences are shown by square brackets.

Table 59

The Effect of Hypervitaminosis D_2 on Placental Wet Weight, Ash Weight, Calcium and Phosphorus Content (4389)

Treatment	No. of placentae	Wet weight (mg)	Ash weight (mg)	Ca (mg)	P (mg)
1 ml olive oil	15	437 ± 10.4[a]	3.6 ± 0.33	0.14 ± 0.02	0.71 ± 0.03
4,000 IU	12	460 ± 10.6	4.5 ± 0.37	0.19 ± 0.04	0.87 ± 0.05
20,000 IU	12	336 ± 16.0	3.8 ± 0.17	0.17 ± 0.01	0.72 ± 0.04
40,000 IU	24	331 ± 15.0	2.8 ± 0.30	0.11 ± 0.02	0.62 ± 0.10

[a]Mean ± SE.
Significant statistical differences are shown by square brackets.

The authors concluded that their findings suggested D_2 may pass through the placental barrier and directly alter the mineral composition of fetal bones.

4. In 1969, Kudo (3327) studied the effect of D deficiency on the sexual cycle and the morphological transformation of the pituitaries, adrenals, ovaries and uteri of white rats (Wistar strain females, mature 120 g with normal sexual cycles, and immature 40-50 g, closed vaginas at birth). D_2 was administered daily for 20 days by injection at various doses (see original paper for experimental details).

Similar experiments by Oniwa (4382) and Chinone (1024) are abstracted on p. 222 and p. 146. Kudo found his results in agreement with Oniwa's report that D reinforced the estrogen effect.

Other findings in this experiment were that in D-deficiency:

a. The rate of pregnancy and the survival rate of the fetus were reduced.

b. The ovary and uterus were also affected, the former atrophied and the latter reduced in weight.

c. The anterior pituitary atrophied with a decrease in number of α-cells.

d. A temporary functional acceleration of the adrenal cortex occurred at the onset of the experiment but later the function deteriorated.

e. There was marked atrophy of the ovaries.

f. The endocrine gland showed functional acceleration with a 100 IU dose but at a dose of 1000 IU a reduction in function was observed.

5. In 1969, Jones et al. (2976) studied the effect of tumor takes and metastases in rats given D_3. The experiments were carried out with 110 male 150 g Sprague-Dawley rats of which 63 were thyroparathyroidectomized (TPT rats) as follows:

Group I: Normal rats (26) administered 0.5 ml saline by s.c. injection.

Group II: Normal rats (51) administered 20,000 IU water soluble D_3 per injection in three s.c. injections on alternate days.

Group III: TPT rats (23) administered 0.5 ml saline.

Group IV: TPT rats (40) administered D_3 as in Group II.

At the end of a week 500,000 Walker sarcoma cells in 0.5 ml saline were injected into a peripheral mesenteric vein of each rat. The results are shown in Table 60 and 61 and Fig. 4. The authors concluded that serum Ca in the 5 mg/100 ml to 16 mg/100 ml range could not be correlated with the incidence of tumor takes.

Table 60

Serum Calcium Levels in Normal (Groups I and II) and Thyroparathyroidectomized (Groups III and IV) Rats Treated with Saline (Groups II and IV) (2976)

Group	I	II	III	IV
Initial serum calcium	-	-	5.26 (±0.14)	5.71 (±0.18)
Serum calcium after treatment	8.68 (±0.18)	11.3 (±0.26)	6.76 (±0.27)	10.87 (±0.34)

Table 61

Incidence of Tumor Takes in Rats After Intramesenteric Venous Injection of Walker Sarcoma. Number of Takes/Total Animals Inoculated (2976)

Group	I	II	III	IV
Liver	10/26 (38.4%)	15/51 (29.4%)	8/23 (34.7%)	13/40 (32.5%)
Mesentery	14/26 (53.8%)	34/51 (66.6%)	14/23 (60.8%)	28/40 (70.0%)

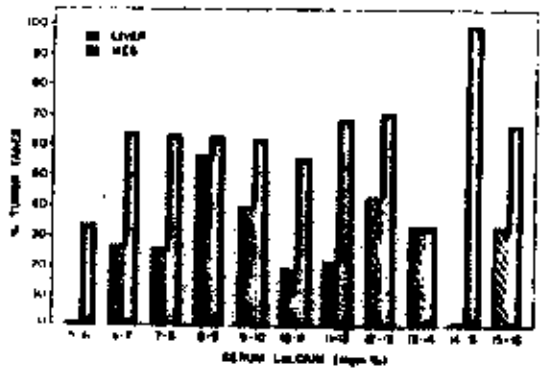


Fig. 4. Serum vs. Tumor Takes (2976)

6. In 1971, Haddad, Jr. et al. (2337) evaluated the metabolism and placental transfer of D_3 and 25-OHD_3 in rats. Female Sprague-Dawley rats (ca. 18 days of gestation) and their non-pregnant female litter-mates (number not given) were injected in the jugular vein with either D_3-^3H (2.9 or 5.8 IU) or 25-OHD_3-^3H (1.7 IU) in 50 to 100 µl ethanol.

It was observed that by 48 hours, almost 20% of the injected doses were found in the fetuses and 12.8% of the fetal radioactivity was water-soluble (See Table 62).

The authors concluded that their data suggested that the placental transfer for D_3 and its active hydroxylated metabolite proceeded at comparable rates.

Table 62

Percent Injected Dose at 48 Hours Following Intravenous ^3H-Vitamin D_3 (2337)

	Water-soluble	Peak I and II	D_3-^3H	25OHD_3-^3H	Polar Metabolites*
Blood					
Nonpregnant	0.59	0.04	1.46	1.28	0.80
Pregnant	0.97	0.08	1.69	0.88	0.24
Fetal compartment	12.83	0.80	3.68	1.54	0.52

	Water-soluble metabolites**	Lipid-soluble metabolites**
Urine		
Nonpregnant	1.08	0.09
Pregnant	0.40	0.09

* As defined by material which was more polar than substance(s) in the peak IV or 25OH-D^3 region on the silicic acid chromatogram.

** As defined following the separation of the phases during the chloroform-methanol extraction.

D. Hamsters

In 1973 Rubin and Levij (4941) examined whether carcinogenesis might be influenced by D_2 and D_3. The model used was carcinogenesis in the hamster cheek pouch induced with 9,10-dimethyl-1,2-benzanthracene (DMBA). The basis for this study was the assumption that competition occurs between DMBA and vitamin D for active sites on the DNA.

Male Syrian golden hamsters (64, 8-10 wks, 70-80 g) were painted on the right cheek pouches with liquid paraffin solutions of DMBA, D_2, D_3, DMBA plus D_2 or DMBA plus D_3 (for details see the original paper) for eight to ten weeks.

The experimental results are summarized in Table 63. No tumors or other pathological changes were found in the animals treated with D_2 or D_3 only. An additional finding was that animals receiving D_2 or D_3 only lost weight (Table 64).

The authors postulated that the marked inhibition of DMBA carcinogenesis in animals treated simultaneously with DMBA and D_2 or D_2 could be due to the induction of the synthesis of APase/CaBP caused by the D vitamins.

Table 63

Incidence of Cheek Pouch Carcinoma in Hamsters Treated Topically with DMBA or with Combinations of DMBA and Vitamins D (4941)

Treatment	Durations, weeks	No. of animals at start/ survivors	No. of animals with tumor	Total no. of tumors in group	Average no. of tumors per treated cheek pouch
DMBA 0.5%	8	20/13	13	75	5.8
DMBA 0.5% + vitamin D_2 0.8%	8	12/10	2	2	0.2
DMBA 0.5% + vitamin D_3 0.8%	10	10/8	1	2	0.3

Table 64

Changes in Body Weight of Hamsters Treated with DMBA, Vitamin D, or Combinations of Both (4941)

Treatment	Weight at start of study in g (range/ average)	Weight at conclusion of study in g (range/ average)
0.5% DMBA	70-80/76	85-110/94
0.8% vitamin D_2	70-80/76	45-75/61
0.8% vitamin D_3	70-80/76	45-70/54
0.5% DMBA + 0.8% vitamin D_2	70-80/76	85-105/93
0.5% DMBA + 0.8% vitamin D_3	70-80/75	80-100/89

E. Guinea Pigs

In 1937, Schmid (5108) reported producing tumors with irradiated ergosterol in the gall bladders of guinea pigs. The experiment involved a surgical procedure in which a 0.2% linseed oil solution of irradiated ergosterol was both introduced into the gall bladder and injected. Other animals were similarly treated with non-irradiated ergosterol and linseed oil.

Small tumors were found in the gall bladders of the irradiated ergosterol animals but no destructive growth or metastases were seen. The non-irradiated ergosterol group showed no bladder changes.

Another group of similar experiments was carried out using a material isolated from irradiated ergosterol, AT.10, and identified as the calcinosis factor. Tumors similar to those noted in the animals exposed to irradiated ergosterol were found. The author considered these experiments too preliminary for evaluation or conclusion. He reported that similar tests with other animals were in progress.

F. Rabbits

1. In 1966, Friedman and Roberts (1947) performed experiments with rabbits to investigate the possibility that the association of supravalvular aortic stenosis (SAS) with idiopathic hypercalcemia in infancy might be due to a maternal D excess or a derangement in D metabolism.

A comparison was made between eight controls and 23 experimental females given large D doses (1.5, 2.5, 3.5 or 4.5 million units of activated

ergosterol in cottonseed oil injected i.m. daily from day of observed copulation) with respect to the extent D was transmitted across the placental barrier at the time of delivery and to the presence of anatomical change in the ascending aorta both at delivery and at intervals up to three months of age when all remaining animals were sacrificed. All offspring received 250 units of D daily in their diet until death or sacrifice.

The observations were:

a. At the time of delivery, both the mothers receiving D and their offspring had blood levels of D seven or eight times normal. The offspring also had slightly elevated blood calcium levels (Table 65).

b. Control mothers showed no gross or microscopic abnormalities of the hearts or aortas.

c. Does receiving 2.5, 3.5 or 4.5 million units D per day died within two months and either aborted or delivered macerated fetuses. Their aortas all showed advanced changes of medial degeneration with irregular depressions in the intimal wall, focal calcium deposits and necrosis.

d. Mothers receiving 1.5 million units per day showed less severe changes. The aortas of 11 newborns out of 18 were normal but showed an exaggerated invagination or plica at the upper margins of the sinuses of Valsalva. Three showed no change and four each from different litters had a prominent annular protrusion at the same supravalvular level resulting in a significant narrowing of the luminal circumference.

e. Four out of ten young rabbits dying between two and 20 days of age showed supravalvular abnormalities; two of these were the annular circumferential type.

f. The aortas of six surviving animals sacrificed at three months showed generalized irregularities of the wall with degeneration and calcification of the media.

The authors noted that:

a. In a significantly higher percentage of rabbits born to mothers given D during pregnancy (as compared to controls), there was exaggerated prominence of the normal plica without apparent narrowing of the lumen.

b. Eight offspring exposed <u>in utero</u> to high levels of D and to the maternal biochemical products of excessive D administration showed pathological abnormalities confined to the supravalvular aortic wall.

c. Six of the eight above showed aortic lesions seriously impinging on the lumen and demonstrating essentially all the histological features of the SAS syndrome as seen in man.

The authors concluded that:

a. In their study D crossed the hemochorial placenta of the rabbit.

Table 65

Results of Analysis of Serum of the Mothers and Offspring (1947)

	Mothers		Offspring	
	Controls	Given vitamin D (1.5 million units)	Controls	Mothers given vitamin D (1.5 million units
Calcium (mEq/L)	7.34 ± 0.24 (SD)	6.88 ± 0.26 (SD)	4.8 ± 0.11 (SD) P<0.05	5.55 ± 0.24 (SD)
Phosphorus (mg %)	4.5 ± 0.75	5.7 ± 0.52		
Alkaline phosphatase (King-Armstrong units)	1.76 ± 0.55	1.15 ± 0.16		
Cholesterol (mg %)	47.3 ± 3.4	56.3 ± 3.9		
Total protein (g %)	4.3 ± 0.77	4.8 ± 0.33		
Albumin (g %)	0.80 ± 0.27	0.87 ± 0.30		
Vitamin D assay (units/100 ml)	310 ± 268 P<0.001	2190 ± 382	150 ± 138 P<0.001	1290 ± 359

b. The vascular toxicity of D could be transmitted across the placenta.

c. An *in utero* derangement in D metabolism on the part of the mother or fetus or both may have been responsible for SAS, especially when the latter was associated with infantile hypercalcemia.

2. Because children with SAS have a characteristic craniofacial appearance and many abnormalities of dentition, in 1969 Friedman and Mills (1950) explored the relationship between exposure to excessive amounts of D during pregnancy and the development of the craniofacial complex and dentition.

Pregnant white New Zealand test rabbits (15) were fed a stock diet and given a total of 750,000 units of D_2 (activated ergosterol in cottonseed oil) intramuscularly in divided doses until delivery. The controls were offspring of six pregnant females receiving cottonseed oil only and 15 given no supplement at all.

It was observed that:

a. The average weight of each test offspring was significantly less than the controls ($P < 0.01$).

b. The occurrence of spontaneous death in the test offspring was significantly greater than the controls ($P < 0.001$).

c. In offspring less than seven days old, the skulls as compared to the controls showed premature closure of the saggital, coronal and lambdoidal sutures.

d. In the less than seven-day old offspring, striking differences in dentition were noted as compared to controls: severe enamel hypoplasia in 95% and a prominent anterior crossbite in 66%.

e. Additional abnormalities were noted in the 15 surviving test offspring sacrificed at 90 days: strabismus was present in 80%; buphthalamos in 30%; the ears were 8 to 10 mm shorter than controls; half had a deep notch of the apical portion of the pinna of one or both ears and in addition to the dentition abnormalities noted above in the neonates, there was partial anodontia and an altered platal contour.

The authors concluded that these experimental observations suggested that the cranial, facial and dental peculiarities as well as the aortic lesion of the SAS syndrome might have been related to a derangement in D metabolism during pregnancy.

G. Dogs

In 1931 Taylor *et al.* (5714) devised experiments to compare the effects of irradiated ergosterol and parathyroid hormone (PTH) overdosage.

a. In the first experiment the influence of irradiated ergosterol on parathyroid tetany was studied by performing a thyroparathyroidectomy on dogs. Severe tetany developed in several of the dogs. Oral

administration of 1-2 ml of irradiated ergosterol produced a prompt and complete cure. The authors postulated that in large doses irradiated ergosterol was as effective an anti-tetanic agent as PTH.

b. In the second experiment animals were more completely operated on so as to remove all possible presence of parathyroid tissue. In these animals irradiated ergosterol was not effective in appreciably improving tetany. Overdosage symptoms appeared when the dosage was continued for two weeks. However, when compared to the dogs in the first experiment (ordinary operation), these dogs showed a greater resistance to overdosage, succumbing in 300 to 360 hours as compared to 168 to 212 hours for the first group.

c. When in a third experiment the effects of large doses of irradiated ergosterol to normal adult dogs (about 40) was compared with the effects of PTH, it was observed that the symptoms were similar to those of PTH overdosage. The findings with six dogs are summarized in Table 66.

The authors noted that it was never possible to distinguish, either by gross or microscopic examination, the effects of PTH overdosage from those caused by irradiated ergosterol. The symptoms and post-mortem findings in the blood were also indistinguishable and the chemistry of the blood was affected in an almost identical manner by either substance. The effects of both substances on Ca and P metabolism were also very similar.

II. Human

1. In 1935 Lewis (3501) tested the relative effectiveness of crystalline D given in milk or in corn oil to rachitic infants. The study was carried out with a total of 36 rachitic infants for a maximum of eight weeks as follows:

a. Nine received 243 USP units daily of D in six drops of corn oil.
b. Nine received 243 USP units daily in milk.
c. Eight received 122 USP units daily in milk.
d. Ten received 2,430 USP units daily in drops of oil.

The results are shown in Table 67. They show that:

a. All the rachitic children receiving 243 USP units in milk showed definite healing after four weeks whereas only three of those receiving the same dose in corn oil showed healing.

b. At the end of six weeks in all those rachitic infants receiving D in milk, healing had progressed, whereas three of the eight infants receiving D in oil showed no healing.

c. At the end of eight weeks the rachitic process became worse for two infants receiving D in oil.

d. In one case in a baby who received 243 USP units daily in oil for four weeks, the rachitic process advanced; then when this infant was

Table 66

THE ADMINISTRATION OF EXCESSIVE DOSES OF IRRADIATED ERGOSTEROL (10,000 D) TO NORMAL DOGS

Animal No. and Sex	Weight kilos.	Dosage c.c. Total	Daily per animal	Daily per kilo.	Equivalent in 250 D per	x maximum* therapeutic dose per kilo.	Dates Administration commenced	First symptoms	Death	Survival time in hours	Serum calcium mg. per 100 c.c.	Post-mortem findings
26 male	7.2	7	2	0.29	11.6	98	Mar. 7, 4 p.m.	Mar. 9	Early a.m. Mar. 10	84 approx.	18.3	Lungs, stomach and upper intestinal tract, haemorrhagic. Large quantity of blood in stomach and bowel.
28 female	14.2	7	2	0.15	6.0	50	Mar. 12, 10 a.m.	Mar. 13	Mar. 16 about 12 a.m.	84	19.5	Essentially the same as above. Gastro-intestinal haemorrhage more intense mucosa of stomach uniformly a deep chocolate colour resembling liver tissue.
39 female	16.1	5	0.8	0.068	2.4	20	Mar. 24, 4 p.m.	Mar. 26	Mar. 28 in p.m.	108 approx.	----	Typical.
40 male	12.5	3	1	0.08	3.2	26	Mar. 27, 4 p.m.	Mar. 28	Mar. 29 p.m.	60 approx.	17.3	Usual signs, but exceptionally intense.
32 male	4.6	2	0.4	0.09	3.6	36	Mar. 20	Mar. 27	Mar. 29	228	18	Unusually severe reaction in lungs and gastro-intestinal tract.
42 female	16.0	6	2	0.12	4.8	40	Apr. 3	------	Apr. 6 p.m.	86 approx.	----	Typical.

*The maximal therapeutic dose is taken as 0.6 c.c. (20 drops) per 5 kilo infant, i.e., 0.12 c.c. per kilo.

Table 67

COMPARISON OF HEALING, AMONG 36 RACHITIC INFANTS, BROUGHT ABOUT BY CRYSTALLINE VITAMIN D INCORPORATED IN MILK OR IN CORN OIL

Case	Age (Mo.)	Date Begun	Menstruum in which crystalline vitamin D was incorporated	No. of Rat units (Steenbock) given	Roentgenologic Rickets: At Onset	Healing After 4 wk.	Healing After 6 wk.	Healing After 8 wk.	Healing After 10 wk.	Comment
C.M.	8	1/23	Milk+	45	Moderate	+	++	+++	++++	Porto Rican
B.G.	6	1/25			Moderate	+	++	++	+++	Porto Rican
F.S.	13	1/26			Moderate	0	0			Negro
E.D.	6	2/7			Slight	+	++	++++		Italian
G.N.	8	2/18			Moderate	+	++	++	+++	Porto Rican
C.W.	22	2/17			Marked	++	++	+++		Negro
G.B.	8	2/21			Moderate	++	+++	++++		Negro
J.H.	6	2/24			Mild	0	0	0 (worse)		Negro
V.L.	6	1/13	Milk‡	90	Slight	++	+++	Normal		Italian
S.Q.	4	1/13			Moderate	++	+++	++++	++++	Porto Rican
J.A.	5	1/13			Slight	+++	++++	Normal		Negro
R.L.	5	1/19			Moderate	+++	Normal	Normal	Normal*	Negro
C.H.	20	1/25			Marked	+++	++++	++++	Normal	Negro—1/25: calcium, 9.8 mg.; phosphorus 3 mg. 2/23: calcium, 10.6 mg.; phosphorus, 5 mg.
K.S.	4	2/22			Slight	++	+++	+++	Normal	Negro
B.C.	7	3/2			Moderate	+				Porto Rican
F.S.	15	3/17			Moderate	+	++			Negro
B.R.	6	3/17			Moderate	++	+++			This infant had received 90 units of crystalline vitamin D in oil for one month, and no healing resulted

+Denotes slight healing; ++, moderate healing; +++, marked healing; ++++, healed rickets.
*X-ray picture taken at 13 weeks.
†One liter contained 60 units.
‡One liter contained 120 units.

Table 67 (cont'd)

Case	Age (Mo.)	Date Begun	Menstruum in which crystalline Vitamin D was incorporated	No. of Rat units (Steenbock given	Roentgenologic Rickets: At Onset	Healing After 4 wk.	Healing After 6 wk.	Healing After 8 wk.	Healing After 10 wk.	Comment
T.R.	5	2/4	Oil*	90	Moderate	++	+++	+++	++++	Porto Rican
S.L.	7	2/12			Slight	?	+	++		
B.M.	4	2/14			Slight	+	++		++	Negro
B.R.	5	2/16			Slight	0 (worse)				Negro
V.B.	14	2/16			Marked	+	++	++	+++	Negro
D.R.	7	2/21			Moderate	0	0	0 (worse)	0	Negro
C.Q.	5	2/23			Slight	?	+	++	+++	Porto Rican
C.B.	8	2/28			Moderate	0	0	0 (worse)		Negro
T.B.	8	3/19			Moderate	0	0			Negro
J.B.	3	2/16	Oil**	900	Slight	++		Normal	Normal	Negro
P.G.	6	2/9			Slight	++	++++			Italian
J.B.	6	2/16			Slight	++	+++	+++	++++	Negro
T.B.	6	2/16			Slight	++	++++	Normal		Negro
C.H.	6	2/16			Moderate	++				Negro
R.P.	17	2/17			Marked	+				Negro
H.B.	4	2/17			Slight	++	+++	++++	Normal	Negro
J.D.	7	2/17			Moderate	++	+++	+++	++++	Negro
N.E.	3	2/17			Slight	+	++	+++		2/15: calcium, 7.2 mg.; phosphorus, 5.3 mg. 3/13: calcium, 19.3 mg.; phosphorus, 6.1 mg.
J.D.	24	1/30			Marked	+	+	+	++	Negro: at 12 weeks healing ++

*One drop contained 15 units.
**One drop contained 150 units.

switched to the same dose in milk for four weeks, definite healing took place.

The author concluded that crystalline D was more effective as an antirachitic agent when administered in milk than when given in corn oil.

2. In 1936 Lewis (3502) investigated the dosage of crystalline D necessary for rickets prevention and the influence of the medium on the effectiveness of the antirachitic dose. There were 441 infants ranging in age from two weeks to five months placed in eight groups as shown in Table 68. (A propylene glycol solution of the crystalline D was used for uniform distribution in the milk). Of the 355 infants followed through to the end, 55% were Negro (188), and the majority of the rest were of Puerto Rican and Italian extraction. The distribution of Negro infants among the eight groups is shown in Table 69.

The results of the investigation are shown in Table 70. Some notable observations were:

a. Infants receiving 145 USP units of crystalline D in milk (Group 1), developed rickets less frequently than those receiving a similar dose in corn oil or propylene glycol. The incidence of rickets in group 1 (145 units in milk) was 5.1%; in group 2 (145 units in corn oil), 14.6%; and in group 3 (145 units in propylene glycol), 13.6%.

b. Infants receiving 290 USP units crystalline D in milk (group 4) also showed a lower incidence of rickets than those receiving the same dose in corn oil (group 5) or propylene glycol (group 6). The incidence of rickets in group 4 was 1.9% in group 5, 9.6%; and in group 6, 11.1%.

c. No infants in either groups 1 or 4 (145 and 290 units D in milk) developed moderate or severe rickets, whereas two in group 2 and three in group 5 (145 and 290 units D in corn oil) and one in group 3 and one in group 6 (145 and 290 units D in propylene glycol) developed the more severe form of rickets.

d. Only one infant, a Negro, in group 7 (1,450 USP units D in corn oil), developed a mild form of rickets.

e. The eight infants of the 22 controls, (group 8) who developed evidence of rickets (as determined by X-ray examination) were all Negro.

f. Of the total number of infants in the study receiving D (groups 1 through 7) who developed rickets (27 infants), 21 (78%) were Negroes.

The author concluded that crystalline D administered in milk was more effective in preventing rickets than when administered in corn oil and propylene glycol.

Table 68

Infant Groups (3502)

GROUP 1	Infants receiving 145 USP units of crystalline vitamin D in milk.
GROUP 2	Infants receiving 145 USP units of crystalline vitamin D in corn oil.
GROUP 3	Infants receiving 145 USP units of crystalline vitamin D in propylene glycol.
GROUP 4	Infants receiving 290 USP units of crystalline vitamin D in milk.
GROUP 5	Infants receiving 290 USP units of crystalline vitamin D in corn oil.
GROUP 6	Infants receiving 290 USP units of crystalline vitamin D in propylene glycol.
GROUP 7	Infants receiving 1,450 USP units of crystalline vitamin D in corn oil.
GROUP 8	Infants serving as controls.

Table 69

The Distribution of Negro Infants as Well as Breast-Fed, Breast- and Bottle-Fed, and Bottle-Fed Infants Among the Various Groups (3502)

Group	Total Number of Infants in each Group	Number of Negro Infants	Percentage of Negro Infants in each Group	Breast-Fed Infants	Breast- and Bottle-Fed Infants	Bottle-Fed Infants
1	58	33	57	--	--	58
2	41	24	58	11	17	13
3	44	24	55	16	17	11
4	51	27	53	--	--	51
5	52	30	58	20	20	12
6	45	26	58	19	14	12
7	42	21	50	14	7	21
8	22	13	59	8	6	8

Table 70. Comparison of the Incidence of Rickets Among Infants Receiving "Crystalline Vitamin "D" Incorporated in Milk, Corn Oil, or Propylene Glycol (3502)

Groups	Number of units (U.S.P.) of crystalline vitamin D	Menstruum in which crystalline vitamin D was incorporated	Number of infants in each group	Average age at beginning of study (months)	Average gain in weight per month during study (lbs.)	Number of infants who developed rickets	Degree of Rickets			Percentage of infants who developed rickets
							Mild	Moderate	Severe	
1	145	Milk*	58	3.3	1.4	3	3	0	0	5.1
2	145	Oil+	41	2.7	1.3	6	4	1	1	14.6
3	145	Propylene glycol	44	2.1	1.3	6	5	0	1	13.6
4	290	Milk‡	51	3.2	1.4	1	1	0	0	1.9
5	290	Oil§	52	3.0	1.4	5	2	2	1	9.6
6	290	Propylene glycol¶	45	3.0	1.2	5	4	1	0	11.1
7	1,450	Oil#	42	3.1	1.3	1	1	0	0	2.4
8	Controls		22	2.6	1.1	8	3	4	1	36.3

*One quart contained 166 U.S.P. (revised) units.
+One drop contained 20.8 U.S.P. (revised) units.
‡One quart contained 332 U.S.P. (revised) units.
¶One drop contained 41.6 U.S.P. (revised) units.
#One drop contained 208 U.S.P. (revised) units.

3. In 1937 Drake (1550) observed the differences in the antirachitic value of various amounts of cod liver oil, viosterol, irradiated fresh milk, irradiated evaporated milk, irradiated cholesterol, irradiated yeast and a mixture of fish liver oils of high potency. For a total of five months (October thru April), over a period of three years, 1228 infants were involved in this study. These were children of British and Northern European stock in Toronto, Canada, who were artifically fed with diluted cow's milk with added carbohydrate. The experimental protocol and results are shown in Table 71. Roentgenograms of the wrists were used to determine rickets. The maximum degree of rickets observed in the controls (no D given) is shown in Table 72.

Some of the observations were:

a. Of the 244 controls (no D), 137 had a moderate or marked degree of rickets, with 21% of these showing an actively advancing rachitic process at the end of the five-month period.

b. The daily administration of 500 USP units of D as irradiated yeast was slightly more effective than were the varying amounts of viosterol used.

c. The antirachitic effect of a very small amount of cod liver oil (one-half teaspoonful, 150 USP units) was as great as that provided by one to three teaspoonsful (Table 72). Judged on the basis of rat units, equally good results were obtained with cod liver oil, percomorph liver oil and irradiated cholesterol.

d. A daily dose of 95 USP units of D in the form of irradiated fresh milk was about equal to that obtained with 150 USP units in the form of the other administered substances. There was no evidence that this extremely small amount was less effective in preventing rickets than were the larger amounts usually given.

e. The daily administration of 300 USP units as irradiated cholesterol or 500 USP units as irradiated yeast resulted in a rapid healing of moderate or severe rickets.

Saelig (5216) commenting on this study noted that from Drake's data, it appeared that a significant number of children of British or Northern European origin require little or no more D than is obtained from nonfortified foods and from sunshine.

4. In 1942, Reynolds (4809) reported on the beneficial effect of D therapy in arthritic patients and the comparative toxicity of two different types of activated ergosterol compounds. He concluded from a review of the literature that:

a. Contradictory findings as to the beneficial vereus the toxic effects of administration of large doses of D indicated that various workers were using different types of preparations.

Table 71

Development of Rickets in Infants, Aged 13 Months and Younger, Who Received Different Amounts of Vitamin D in Various Forms Throughout Five Winter Months ( )

Year of Observation	Source of Vitamin D	No. of USP Units of Vitamin D Given Daily	No. of Infants	Age, Months: Less Than 1	1	2	3	4	5	6	7	8	Percentage of Infants: Maximum Degree of Rickets Subsequent to Initial Examination: None	Extremely Slight	Mild	Moderate and Marked	Actively Advancing Rickets with No Evidence of Healing at end of Period of Observation
1933-34	1 teaspoonful cod liver oil	350	42	0	1	8	5	4	8	3	10	3	81	12	7	0	0
1933-34	2 teaspoonfuls cod liver oil	700	45	0	2	5	8	10	4	7	8	1	75	16	7	2	2
1933-34	3 teaspoonfuls cod liver oil	1,050	50	0	7	5	5	6	9	6	10	2	74	14	8	4	4
1933-34	1-1/4 drops viosterol	270	50	0	9	10	11	4	3	8	3	2	82	16	2	0	0
1933-34	2-1/2 drops viosterol	540	44	1	10	8	10	3	5	2	5	0	68	25	7	0	2
1933-34	5 drops viosterol	1,080	46	0	12	13	2	6	9	1	1	2	78	20	2	0	2
1933-34	10 drops viosterol	2,160	46	1	5	13	7	8	4	4	3	1	76	17	7	0	4
1933-34	20 ounces (592 cc.) irradiated fresh milk	95	71	0	8	16	14	8	8	8	7	2	69	23	8	0	3
1933-34	20-40 ounces (592-1, 184 cc.) irradiated fresh milk	95-190	70	0	0	5	4	13	16	12	11	9	81	16	3	0	0
1933-34	No vitamin D	0	65	1	13	10	7	10	9	9	5	1	55	22	15	8	16
1934-35	10-40 ounces (296-1, 184 cc.) irradiated fresh milk	47-190	102	0	8	14	17	29	21	13	0	0	75	13	12	0	0
1934-35	6-20 ounces (177.6-592 cc.) irradiated evaporated milk	60-196	103	0	19	20	24	17	19	4	0	0	64	19	17	0	0
1934-35	No vitamin D	0	104	1	19	22	30	16	13	3	0	0	47	10	27	16	16
1935-36	Irradiated yeast	500	69	0	0	0	0	13	27	23	6	0	87	4	9	0	0
1935-36	0.43 teaspoonful cod liver oil	150	74	0	13	21	13	17	6	4	0	0	69	19	12	0	3
1935-36	0.75 drops percomorph liver oils	150	59	0	7	11	8	12	11	8	1	1	75	15	10	0	2
1935-36	Irradiated cholesterol	150	77	0	15	12	21	17	10	2	0	0	82	8	10	0	1
1935-36	No vitamin D	0	75	1	12	19	16	16	7	2	0	0	51	16	20	13	31

Table 72

Development of Rickets in Infants Who Received No Vitamin D Throughout Five Winter Months (1550)

Year of Observation	Total No. of Infants	Percentage of Infants				
		Maximum Degree of Rickets Subsequent to Initial Examination				Actively Advancing Rickets with No Evidence of Healing at End of Period of Observation
		No Rickets	Extremely Slight Rickets	Mild Rickets	Moderate and Marked Rickets	
1933-34	65	55	22	15	8	18
1934-35	104	47	10	27	16	16
1935-36	75	51	16	20	13	31
Total, 1933-36	244	50	15	22	13	21

b. A specific finding was that massive doses of irradiated ergosterol caused toxic effects without clinical improvement of arthritic patients, whereas ergosterol produced by the Whittier process (electric discharge-activated heat-vaporized ergosterol) appeared to bring about improvement with negligible or no toxicity.

5. In 1950, Touraine and Zureick (5820) reviewed a number of earlier French reports claiming that D was carcinogenic on the bases of both its molecular structure and clinical observations. The authors concluded that while the evidence did not justify the claims, it did warrant further study.

6. In 1951 Freedman (1920) examined the vaginal changes in menopausal and postmenopausal women given D. The 27 women (39 to 68 years of age) in this study fell into three groups:

a. Five artificially castrated women.
b. Seven who had not menstruated for three or more years.
c. Fifteen who had menstruated within three years.

They were orally administered from 2,250,000 to 2,550,000 units of D for 15 to 17 days as 50,000 unit capsules three times daily. Improvement on an arbitrary vaginal smear scale or an increase in mucoid discharge were considered to be positive results. The observations showed that:

a. Four tests in the first group were positive.
b. Four tests in the second group also were positive.

c. Thirteen positive vaginal smears were found in the last group.
d. The results of 37 tests performed on the 27 patients showed 22 positive: 19 improved vaginal smears, and 3 vaginal mucus elevations. (For similar studies with rats see Oniwa, p. 222, Kudo, p. 150, and Chinone, p. 146).

7. In 1959 Keres (3105) reported observations of hypervitaminosis D made at a children's hospital in the Soviet Union. In one case, a 14-month old child showed clinical symptoms of hypervitaminosis D after receiving a total of six million units of D in four months (62,500 units daily) which was ten times the therapeutic dose. Her serum calcium was 20 mg%.

In another case only twice the therapeutic dose of D for four months (20,833 units daily) resulted in hypercalcemia with a serum calcium of 15.5 mg%. The clinical symptoms included apathy, subnormal growth and frequent vomiting. The ESR was 35 mm per hour. The blood calcium and ESR returned to normal along with general improvement after the D was stopped.

In another child, ten months old, a dose ten times the therapeutic dose over four months (40,000 units daily totaling 3,375,000 units) did not produce clinical symptoms, but x-ray examination showed decalcification in the epiphyses of the tubular bones.

The author pointed out that other studies had shown the absorption of Ca from cow's milk to vary under different conditions: normally, 35% was absorbed; with D, up to 50% was absorbed; and up to 60% was absorbed in idiopathic hypercalcemia.

According to this author, idiopathic hypercalcemia was more likely to appear in infants fed cow's milk in which the calcium content was 4.5 times that of maternal milk. He concluded that:

a. Even insignificant overdoses of D could cause symptoms of hypervitaminosis D to develop.

b. There were wide individual differences among children in their sensitivity to D.

8. In 1963, Kenny et al. (3101) reported a case of idiopathic hypercalcemia of infancy in which elevation of the serum Ca and probable elevation of the serum D level were produced during the recovery phase by administering 400 units of D per day. An unusual aspect of the case noted by the authors was the familial incidence. The patient had a sibling previously diagnosed as having severe idiopathic hypercalcemia. According to the authors, this suggested that some cases of idiopathic hypercalcemia might result from an inborn error of metabolism.

9. In 1964 Bell et al. (0459) studied the effect of D on four patients with sarcoidosis as compared to three normal subjects. Vitamin D therapy with small doses (10,000 IU/day), resulted in excessive absorptions of Ca without abnormally increasing serum antirachitic activity. Prednisone prevented this effect. It was also noted that the incidence of hypercalcemia in patients with sarcoidosis was greater during the summer months when the production of D_3 in the skin by UV radiation was enhanced.

The authors concluded that their results along with those of Hendrix (Clin. Res. 11:220, 1963) who showed that the converse, the hyperabsorption of Ca by patients with sarcoidosis can be diminished by feeding them a diet deficient in D, supported the hypothesis (Anderson et al., Lancet, 2:720, 1954) that the defect in Ca metabolism was a result of intestinal hyperabsorption of Ca caused by an increased sensitivity to D. Prednisone and other carbohydrate active steroids acted by antagonizing this action of D.

10. In 1966 Fraser et al. (1894) reviewed infantile hypercalcemia:

a. The mild form was distinguished from the severe form.

b. Neither form was associated with excessive D intakes by the mother while pregnant.

c. Cardiovascular involvement was part of the severe syndrome, but whether this was sometimes or always was uncertain.

d. In the severe form serum Ca could return to normal while other manifestations persisted, so that many advanced cases would escape diagnosis.

e. Mental retardation, mentioned by all predecessors, was now emphasized.

f. Prognosis was good for the mild form, poor for the severe form.

Treatment proposed by the authors involved reduction of Ca intake to 25-35 g/day, elimination of D from the diet, and protection of the patient from sunlight. Short-term cortisone treatments were also suggested.

The authors stated that in their opinion the classic study by Jeans and Stearns (2993) "does not stand up to modern tests of significance."

11. In 1966 Beuren et al. (0526) presented a clinical report without laboratory data concerning the existence of SAS in an unusually large number of German children (this report is abstracted in detail on p. 126). The case histories of some of the patients showed that the mothers took D during pregnancy. The authors pointed out that in Germany, one massive dose of Vigantol (a vitamin D preparation) in excess of the amount required for rickets prevention was recommended in the sixth and ninth month of pregnancy.

12. A commission of the German Pediatric Society (5153) studied this and other reports and concluded that:

a. Administration of toxic doses of D to pregnant animals induced SAS, along with other vascular injuries, in some of their offspring.

b. When D hypersensitivity was present, every administration of D could contribute to the appearance of this syndrome during pregnancy. Perhaps even the production of D by the body could also contribute in those few individuals who were extremely sensitive.

c. The commission recommended that massive D doses not be given during pregnancy.

13. In 1967, Friedman (1949) reviewed the literature on SAS. Some of the points he raised were:

a. The vascular toxicity of large doses of D had been observed in non-pregnant animals.

b. Information on the effects of induced maternal hypervitaminosis D on the fetus had been limited.

c. There was recent direct evidence for transplacental passage of D.

d. A study with pregnant rabbits suggested that a derangement of D metabolism during pregnancy on the part of the mother, the fetus or both, could be responsible for SAS particularly when this condition was associated with infantile hypercalcemia.

e. The question still to be answered was why there was a variation in D sensitivity between different individuals of the same species as well as between different species.

The author considered the need for detecting pregnancies susceptible to the teratogenic effects of D and related sterols, in order to prevent SAS, sufficient to warrant research on the epidemiologic, genetic metabolic and pathologic information relating to the disease.

14. In 1968, Cooke (1132) reviewed the relationship of infantile hypercalcemia to D. He pointed out that D sensitivity was intrinsically difficult to investigate. He reported the results of several experiments on maternal D intakes:

a. A study of 58 normal pregnancies showed that a 250 IU daily supplemental dose of D plus 1.44 g per day of supplemental Ca resulted in increased bone density in the fetus.

b. Animal studies demonstrated that maternal D had a dose-related effect on the fetus producing bone abnormalities at low toxic levels and death at higher ones.

The author considered various evidence sufficient to conclude that:

a. The more severe form of infantile hypercalcemia had its genesis in utero.

b. The less severe borderline forms probably, but not certainly, stemmed from intrauterine effects.

c. The higher the D intake (among mothers or infants) the higher the incidence of hypercalcemia (from British epidemiological evidence).

15. In 1970 Seelig (5218) reviewed the medical evidence relating D and the various toxic reactions to it reported in the literature. These included: infantile hypercalcemia with its associated renal and cardiac damage as well as mental retardation; supravalvular aortic stenosis or Williams' Syndrome; generalized arteriosclerosis of infancy; renal acidosis; and nephrocalcinosis infantum resulting from symptomless hypercalcemia.

Seelig noted that "evidence has accrued that the amount of vitamin D added to milk (which is enough to prevent, or even to cure rickets in children who require very large amounts) is sufficient to cause renal, cardiovascular and brain damage in those who are hyperreactive". She went on to point out that "since vitamin D is much more active in milk than it is in oil, and since there is evidence that vitamin D_2 is more toxic than vitamin D_3, the addition of 400 units of vitamin D_2 to a quart of milk may provide for more than a safe intake for susceptible infants".

The author therefore recommended that "routine diagnostic tests to detect reactivity to vitamin D, preferably in the first week of life should be developed." However, "until suitable tests are available and widely employed, milk should be made available in two forms; one that is free of added vitamin D, and one that is fortified, preferably at the same price so that economics not play a role in the milk bought."

16. In 1973 Rosen et al. (4900) measured the major circulating metabolite of D_3, 25-OH-D_3 in mothers and their affected premature infants, to examine further the possible role of D_3 in the pathogenesis of neonatal hypocalcemia (NH) of prematurity. Their data suggested to the researchers a direct role of D deficiency as one of several pathogenic factors in NH of prematurity and that nutritional D deficiency might be relatively common in mothers whose pregnancies were likely to end prematurely. They gave no information concerning the ethnic background of the mother-premature pairs studied.

BIOCHEMICAL ASPECTS

I. Breakdown

This section is designed to cover spontaneous changes undergone by a substance in foods and food sources. In the case of substances with D activity, such changes are described in the Chemical Information Section and under Metabolism.

II. Absorption-Distribution

In 1956 Kodicek noted in a review (3208) that D was absorbed efficiently by rats after topical application to the skin.

In 1964 Bell et al. (0459) measured Ca, P and N balances both in four patients with sarcoidosis and high urinary Ca, and in three normal subjects.

When D (form not stated) 10,000 IU/day was given for 12 days, Ca absorption and fecal excretion were unaffected in the normal subjects, while a more positive balance occurred in the patients.

Prednisone given to the patients diminished Ca absorption, whether "spontaneous" or induced by the D.

The authors concluded that patients with sarcoidosis were abnormally sensitive to vitamin D.

In 1966 Thompson et al. (5765) studied intestinal absorption of labeled D_3 0.5-1.0 mg given orally to ten gastrectomized patients, six with osteomalacia.

Four of the six had slightly low absorptions, the fifth had nil owing to a pancreatic defect, and these five had steatorrhea. The sixth had normal absorption and no steatorrhea.

In the four without osteomalacia absorption was normal although two had steatorrhea.

The authors commented that the site of D-absorption was still uncertain, and that physiologic doses could be malabsorbed as a result of partial gastrectomy.

In 1970 Chen et al. (1004) studied the subcellular location of the D_3 or its metabolite associated with transcription in intestinal mucosal cells. They contested the theory that it was associated with nuclear chromatin by showing that relevant chromatin preparations were contaminated with membrane fragments. They concluded tentatively that the D_3 compound was located in the nuclear membrane, and anticipated more information from future studies with the labeled $25,26\text{-}(OH)_2\text{-}D_3$ derivative.

In 1971 Lumb et al. (3607) reported a study of relationships between massive D intakes and serum D values (see also p. 131). They studied both normal subjects and patients with D-resistant kidney-related Ca disorders. They found that:

1. Massive intakes of D did not correspondingly affect serum D values. A case was cited of serum D at 1 IU/ml two years after withdrawal of massive D therapy, when the skin, muscle, fat, and bones had 8-10 times higher values. In another case, a family poisoned accidentally by food cooked in oil containing 5,000,000 IU/g had serum D values of only 66 IU/ml.

2. Decay curves (see original paper) showed elevated serum D for many months after stopping massive intakes. Decrease was rapid at first, but from the fourth to the 47th month the half-life was 16-17 months. Therefore, according to the authors, D must be stored in other tissues, and their observations suggested the muscle as most important.

In 1971 Smith and Goodman (5384) studied the transport and turnover of D_3 in man. Others had already identified 25-OH-D_3, tentatively isolated 1,25-$(OH)_2$-D_3, and tentatively associated these metabolites with the albumin and α-globulin fractions of serum, revealing that the disappearance curve had several components.

Smith and Goodman gave four normal men each 6 μg of labeled D_3 i.v. They found that the label became entirely associated with a protein of density >1.21, which therefore was not a lipoprotein. This was confirmed by uptakes of Ca^{45}. The protein was partly characterized and was found to have a mw of 50,000-60,000; it was slightly smaller and faster-moving, electrophoretically, than albumin.

The authors inferred that this protein transported 25-OH-D_3.

The initial half-life of D_3 in plasma was found to be 12 hours, and its residual level in plasma was less than 1% of that of the label. The D_3 was replaced by 25-OH-D_3 with a half-life in plasma of 19.6 days, and the 25-OH-D_3 accounted for 92% of the label.

In 1971 Haddad et al. (2337) established that both D_3 and 25-OH-D_3 were readily transferred across rat placenta to fetuses (see p. 152).

III. Metabolism and Excretion

A. Metabolism

In 1934 Waddell (6048) established that cholesterol, when irradiated, contained a different provitamin D from the provitamin D present in irradiated

ergosterol. The cholesterol provitamin was more potent against rickets in chicks, and also was probably the main precursor of vitamin D in the body.

The author noted that milk was reported to be a more potent source of D activity than cod liver oil; he inferred from his own work and that of others that both sources contained mainly the cholesterol provitamin, and he concluded that the D factors in milk "may possess virtues still not understood but which may be explained for the moment on the assumption of better absorption, etc."

In 1963 Cuthbertson (1256) reviewed his and others' evidence for a relationship between idiopathic hypercalcemia and D.

In the severe Fanconi syndrome, serum D was raised although intakes were normal. Therefore there must be a "derangement" of either Ca metabolism or D metabolism. The author suggested accumulation of a D metabolite that was active in man but not rats, such as dehydrotachysterol.

However, the mild Lightwood-type cases could not be so explained, since serum Ca was in the normal range.

In 1965 Quarterman (4671) gave D_3 by injection to rats, sheep, goats, rabbits, and a pig, and observed increases of a substance that was not D (chemically or biologically) between 15 minutes and 2 hours later in the adrenal glands, livers, kidneys, and ileums. The authors associated this substance with D but could find no direct evidence that it was a derivative.

In 1966 Fraser and Kodicek (1905) gave (1-^{3}H)-D_3 orally to a 125-g rat and collected lymph hourly from a thoracic duct cannula. Peak concentrations of label were at 2-3 hours, and 43% of the dose had entered the lymph at 12 hours. Up to 2.6% of each fraction was esterified, and the composition of the esters was: stearate 25%, oleate 16%, linoleate 16%, palmitate 31%, remainder total 13%.

The authors remarked that this composition was like that in liver but not in kidney, and they inferred that the small intestine was the site of esterification.

In 1966 Lund et al. (3613) confirmed the report of Fraser and Kodicek (1905) about the in vivo esterification of D. They commented that the function of the esters was uncertain. Storage (as with retinol) was unlikely because the amounts were so small; activity was unlikely because there was so little at low dosage. They concluded that "esters of vitamin D may be only a metabolic curiosity" with no function that was meaningful at the time of writing.

In 1966 Lund and DeLuca unequivocally isolated a polar metabolite of D_3 that they described as Peak IV (3611).

In 1967 Loomis (3575) documented a hypothesis that human interracial differences of skin pigmentation were caused by geographical differences of UV irradiation and mediated by vitamin D.

Pointing to rachitic effects of D-deficiency on the one hand, and to soft-tissue calcification and renal disorders of D-excess on the other hand, he commented that the only endogenous regulator of the amount of D-accumulation in the body seemed to be an adaptive control of its photochemical synthesis in the skin.

This control, the author continued, should be exerted by (a) skin pigmentation and (b) keratinization of the skin stratum corneum to degrees that ideally would restrict endogenous D-synthesis to the physiological range 0.01-2.5 ng/day. There were two phenomena: irreversible pigmentation-keratinization that was latitude-related, and seasonally reversible pigmentation-keratinization restricted originally to northern latitudes.

On the (cited) basis that 1 cm^2 of white human skin synthesizes ca. 6 IU of D/hour, the author reckoned that 400 IU/day would be synthesized by diurnal exposure of 20 cm^2 of an infant's face in northern Europe. In the tropics, a white person exposing 1.5 m^2 for 6 hours would synthesize 800,000 IU. White skin *in vitro* transmitted both 405 and 365 nm wavebands, but Negro skin was opaque to wavelengths below 436 nm. Later studies cited showed an overlap of skin permeabilities to UV between Europeans and Nigerians.

According to Loomis, these data showed that Negro infants, known to be more susceptible to rickets than White infants, would theoretically be D-deficient in northern latitudes, although both dark and fair skins contained similar and adequate amounts of the provitamin D_3. The author then used these considerations to speculate upon the possible evolution of observed grades of skin pigmentation.

Arguments followed (3576). Blois objected that (a) melanin and tyrosinase were distributed through most phyla, but the most primitive animals that contained D were the teleosts; (b) primitive man might have lived in forests, not exposed to sunlight; (c) the range of photosynthetic control given by Loomis was many times narrower than the range he gave for human tolerances to ingested vitamin D; and (d) there were no reports of hypervitaminosis D in

albinos. Blois concluded that Loomis' mechanism could not be the primary regulator of skin color.

Blum, who had been cited by Loomis, pointed out that melanin protected against other effects of UV exposure that did not involve D, such as sunburn and skin cancer.

In rebuttal Loomis stated that (a) CO_2 occurred in bacteria, yet it controlled mammalian respiration; (b) melanin was known to protect the mammalian retina; (c) protoporphyrin occurred in protozoan cytochromes and in mammalian hemoglobins; (d) hypervitaminosis D had been reported in infants receiving less than 1800 IU/day; (e) his hypothesis did not rule out skin cancer and sunburn as agents of evolution together with photoactivation of D; and (f) he gave further data on serum Ca levels and UV penetration of the skin at various latitudes.

In 1967 Horii et al. (4072) gave ^3H-labeled D_3 to rats and isolated a metabolite from their carcasses that was "as active as" D_3 at curing rickets, stimulating Ca transport, and raising blood Ca. Given to D-deficient rats it stimulated intestinal Ca transport within 8-10 hours, contrasted with 20 hours for D_3 itself. The metabolite was identified chromatographically as Peak IV.

In 1968 Fraser and Kodicek (1902) constructed some theoretical 3-dimensional models of cholesterol, D_3, and an intermediate precursor of D_3, in order to find out why D_2 and D_3 were acted on by pancreatic esterifying enzyme. Some very specific conformational requirements had been calculated in studies with cholesterol, and the vitamins D did not have those requirements.

They discovered that although the C-3 OH-group of the vitamins was not linked in the required manner to the rest of the molecule, it occupied the same position in 3-dimensional space as did its counterpart in the cholesterol molecule; had it been linked in the required manner, it would not have occupied that position.

The authors concluded that the position in space was more significant than the linkage to the rest of the molecule. They suggested that therefore the D vitamins should also be substrates for other cholesterol-specific enzymes such as sulfokinase, and that the position of the conjugated triene system was more important than that of the side chain. The principle in these suggestions led in due course to recognition of the hormonal importance of the position in space of the 1α hydroxyl group of the vitamins D (0161, 2688, 2689).

In 1968 Lund, in his Dissertation (3611, only the Abstract is cited), reported the chromatographic separation of some D metabolites.

Peak I yielded D itself on saponification and was inferred to contain esters.

Peak II was not identified, but was stated to be neither 5,6-*trans*-D nor pre-vitamin D.

Peak III was tentatively identified as unaltered D.

Peak IV was described as a polar metabolite that "could be the metabolically active form of vitamin D."

In 1968 Blunt et al (0633) gave 4 hogs daily supplements of D_3 250,000 IU for 26 days, and recovered 1.3 mg of a pure metabolite from 6.8 liters of their plasma. They found this to be the major active component of their Peak IV fraction, and up to 1.4 times more active than D_3 at curing rickets in rats (0633). By a combination of UV, mass spectra, nmr spectra, and gas-liquid partition chromatography, they identified the pure metabolite as 25-hydroxycholecalciferol (25-OH-D_3).

In 1969 DeLuca (1453) reviewed advances in knowledge of D, noting the importance of Kodicek's observation in 1956 that D was excreted, not intact, but as metabolites.

The author described his synthesis of D_4, ergosterol acetate labeled at C-22,23. He reviewed the isolation, characterization, and synthesis of 25-OH-D_3, which at that time he believed to be the metabolically active form of the vitamin. He stated that 25-OH-D_3 was accumulated by the nuclear membrane of the intestinal mucosa, where it induced transcription of specific mRNA for a Ca transport protein.

In 1969 Avioli et al (0263, paper delivered the previous year) investigated the theory that azotemia with bone demineralization in kidney failure might reflect a redistribution of Ca, and that the observed insensitivity to vitamin D might be limited to the intestinal mucosa.

Labeled D_3 was given to eight young adult patients with kidney failure, and findings suggested:

1. Defective conversion of D_3 to 25-OH-D_3, and
2. Accelerated destruction of D_3.

Adult rats then had one kidney removed and, together with controls, were killed 72 hours after injection with labeled D_3. Blood, urine, and

feces were analyzed. Findings indicated a defect in D_3 metabolism. The authors suggested that the defect was in the induced synthesis of CaBP.

In 1969 Ponchon and DeLuca (4603) isolated 12 metabolites of D_3 by column chromatography. They found over 80% of the metabolites in Peaks III, IV, Va, and Vb. Peak I was identified as esters. Peak II was reported as not yet characterized. Peak III was D_3 unchanged. Peak IV was identified as 25-OH-D_3. Peaks Va and Vb from plasma and skeleton were inactive when bioassayed, but the same peaks from kidney contained 37% of the total D-activity. These and the remaining peaks, which were inactive, were not characterized.

In 1969 Suda et al. (5588) announced the isolation of 314 µg of a polar metabolite of D_2 from the blood of 4 pigs given 500,000 IU D_2 per day for 26 days. They identified this as 25-hydroxyergocalciferol (25-OH-D_2) by UV spectra, gas-liquid partition chromatography, nmr spectra, and mass-spectra. They found 25-OH-D_2 to have 1.5 times the rat antirachitic potency of D_3 or D_2.

In 1969 Drescher et al. (1559) investigated the site where chicks discriminated metabolically against D_2. They found that chicks elaborated polar metabolites, defined chromatographically as peak IV, from both D_2 and D_3. However, the D_2 metabolite was barely active in chicks, while the D_3 metabolite was more active than D_3 itself. On bioassay in rats, both metabolites were equally and fully active.

The authors concluded that the chick discriminated against D_2 at the metabolic level of the peak IV metabolites, and speculated that they might do so by more rapid degradation of the D_2 metabolite.

In 1969 Ponchon et al. (4598) reported that when the livers of rats were isolated from their circulations, labeled (1,2-^{3}H) D_3 introduced into the circulations was not converted to 25-OH-D_3.

They concluded:

1. that the liver was the major and perhaps only site for conversion of D_3 to 25-OH-D_3, and
2. that osteodystrophy and higher D requirements associated with hepatic insufficiency might be explained by this.

In 1970 Ponchon and DeLuca (4597) prepared 25-OH-D_3 labeled with H^3 at C26 and 27, and found that 2 hours after i.v. injection into chicks it

was further metabolized into two substances, identified as Peaks V and VI. They inferred from relative potencies that these products reflected control of D effects by metabolic inactivation of 25-OH-D_3.

In 1970 Suda et al. (5585) reported that 26-OH-D_2 was 1.5 times more effective than D_2 or D_3 at curing rickets in rats: it induced intestinal transport of Ca and mobilization of Ca from bone, and acted in both systems more rapidly than D_2.

They concluded that the 25-OH-D_2 was the "circulating active form of D_2 and that all of the D vitamins and dihydrotachysterols must be hydroxylated at C-25 in order to become active. Although 25-OH-D_2 "does not appear quite as effective as" 25-OH-D_3, the authors emphasized that their comparisons were preliminary.

In 1970 Suda et al. (5586) gave eight pigs each 250,000 IU D_3 per day for 28 days and isolated 40 micrograms of a metabolite that was "unequivocally" 25,26-$(OH)_2$-D_3.

This was found significantly active only in the intestine, and the authors commented that the 21,25 metabolite was, by contrast, active only in bone. The question whether these metabolites were intermediates or endproducts was being studied.

In 1970 Cousins et al. (1181) found that when rats were given 0.025 µg of labeled 25-OH-D_3 by intrajugular injection and were killed at intervals from 3 minutes to 3 hours later, 50% of the label accumulated in the nuclear fraction of the intestinal mucosa. When 0.25 µg was given, 25% of the label accumulated there.

The 25-OH-D_3 was converted in vivo to two metabolites, identified chromatographically as peak V and a more polar metabolite, peak VI. Within 30 minutes 72% of the label was in peak VI. Two hours after the dose the balance began to alter, and by the eighth hour 58% of the label was in peak V.

The authors concluded that peak VI was "probably" the precursor of peak V. They inferred from the paucity of label in the cytoplasm that both metabolites originated in the nucleus, and therefore should be relevant to the Ca transport mechanism.

In 1970 Fraser and Kodicek (1903) gave chicks doubly labeled D_3 (4-^{14}C; 1-^{3}H) and isolated from their intestinal cell nuclei a more polar metabolite than 25-OH-D_3 with triple the biological activity. They stated that their

findings were compatible with oxygen insertion at C-1. Other experiments established that this metabolite was elaborated in the kidney and nowhere else. The authors suggested therapeutic uses for this metabolite in disorders of Ca metabolism involving losses of kidney function.

In 1971 Gray et al. (2227) confirmed the finding of Fraser and Kodicek (1903) that nephrectomy prevented the appearance in the gut of the polar metabolite of 25-OH-D_3.

1. The authors also reported that the metabolite, as produced by kidney homogenates in vitro, was chromatographically identical to that found in the intestine.

2. They also reported that uremia did not prevent its elaboration in rats.

In 1971 Holick et al. (2691) gave D-deficient chicks (1-^{3}H)-D_3 and 24 hours later they isolated from their intestines, purified, and identified a metabolite more polar than 25-OH-D_3. The metabolite, 1,25-dihydroxycholecalciferol, or 1,25-$(OH)_2$-D_3, was identified by specific chemical reactions, and by UV and mass spectra.

In 1971 Norman et al. (4302) reported the mass spectra of D_3, 25-OH-D_3, and 1,25-$(OH)_2$-D_3 isolated from chick intestines (see Fig.).

In a further 1971 report Holick et al. (2693) gave more details of their methods and inferences. They emphasized that the stereochemical position of the OH at C-1 had not been finally established, but gave reasons for inferring that it was α.

The authors showed that 1,25-$(OH)_2$-D_3 "or a further metabolite thereof must be the metabolically active form of vitamin D in the intestine".

However, it was not more active than 25-OH-D_3 at mobilizing bone Ca, and was not produced in bone cultures from 25-OH-D_3. Therefore its status in bone was unclear.

The authors concluded that this metabolite might have uses in treatment of D-resistant renal osteodystrophy and familial hypophosphatemia.

In a further report of 1971 Omdahl et al. (4375) found a concentration of 1,25-$(OH)_2$-D_3 in chick intestines of 13 pmoles/chick, regardless of D

intake. Ca transport responded faster and more strongly to 375 pmoles of $1,25\text{-(OH)}_2\text{-D}_3$ than to the same of 25-OH-D_3.

However, 25-OH-D_3 was more than twice as effective against rickets in the rat. The authors attributed this to longer half-life, and reported a similar comparison of effects on serum Ca.

They concluded that $1,25\text{-(OH)}_2\text{-D}_3$ was the specific active metabolite for intestinal Ca transport, but could not decide between this and 25-OH-D_3 for specificity in bone.

In 1971 Norman et al. (4301) injected D_3 into the hearts of chicks with rickets and found most of the label 15 hours with three polar metabolites, 25-OH-D_3, $1,25\text{-(OH)}_2\text{-D}_3$, and an unidentified metabolite designated 4C. The 25-OH metabolite was mostly in the intestine, and the $1,25\text{-(OH)}_2$ metabolite mostly in the plasma. In further experiments these two metabolites were identified in chick skeleton, laying-hen uterus, rat, rabbit, frog, and monkey.

In 1971 Haddad et al. (2337) injected pregnant rats with labeled D_3 and found about 20% of the dose in the fetuses after 48 hours. Water soluble metabolites carried 12.8% of the label in the fetuses. The dose was more concentrated in the fetuses than in the mothers.

When labeled 25-OH-D_3 was injected, 2% of the dose was found in the fetuses after one hour, together with smaller amounts of more polar metabolites. When labeled D_3 was given, the maternal/fetal ratio was similar but the recovery of label from blood was smaller than after the 25-OH-D_3.

The authors concluded that both of these compounds were readily transferred across the placenta, but cautioned that their data did not reveal the extent, if any, to which the fetuses had metabolized either D_3 or 25-OH-D_3.

In 1973 Holick et al. (2688) synthesized $1\alpha\text{-OH-D}_3$ from cholesterol. Previously they had synthesized $1\alpha,25\text{-(OH)}_2\text{-D}_3$ from homocholenic acid. Comparisons of these two synthetics revealed that:

1. They were approximately equipotent for intestinal transport of Ca and for its mobilization from bone in both normal rats and rats without kidneys. However, while both compounds produced responses at 62.5 pmole, when doses were raised from 625 to 1250 pmole the $1\alpha,25\text{-(OH)}_2\text{-D}_3$ produced a further increase, and the $1\alpha\text{-OH-D}_3$ did not.

2. The $1\alpha\text{-OH-D}_3$ was found to be easier and cheaper to synthesize.

3. Although 5,6-*trans*-D_3 was also easy and cheap to synthesize, and induced Ca transport and mobilization in rats without kidneys, it was 50-100 times less potent than either 1,25-$(OH)_2$-D_3 or 1α-OH-D_3.

4. Evidence was needed whether or not 1α-OH-D_3 was further hydroxylated in vivo. On the one hand, the time lag to maximal response was the same as with 1α,25-$(OH)_2$-D_3. On the other, the response to 1α-OH-D_3 had a lower peak and lasted longer.

The authors concluded that synthetic 1α-OH-D_3 had a potential usefulness in medicine.

In a further report in 1973 Holick et al. (2689) discussed the synthesis of further analogs of D_3.

1. From previous work they noted that 180° rotation of the A ring of D_3 brought the 3β-OH into the place occupied by the 1α-OH of 1,25-$(OH)_2$-D_3; thus 5,6-*trans*-D_3 induced both transport and mobilization of Ca in rats without kidneys, while its 25-OH derivative induced only transport.

2. Therefore, they synthesized and tested two other compounds with the 3β-OH similarly placed. These were iso-D_3 and isotachysterol$_3$, and 25-OH-isotachysterol$_3$ was also synthesized. All three had both functions in normal rats; surprisingly, isotachysterol$_3$ but not iso-D_3 was active in rats without kidneys. Also surprisingly, the 25-OH-isotachysterol$_3$ was active.

The authors were surprised because, if the 1α location of 1,25-$(OH)_2D_3$ were the active site, iso-D_3 should have been active, and 25-OH-isotachysterol$_3$ inactive, in rats without kidneys. They advanced a number of theoretically possible explanations to be tested.

They concluded that "in any case" isotachysterol$_3$ had been revealed as another active isomer of D_3 with potential usefulness in the treatment of renal osteodystrophy. They stated that its synthesis from D_3 was as simple as that of the 5,6-*trans* isomer, and unlike that isomer, was quantitative. Thus it would be much easier to purify.

In 1973 Omdahl and DeLuca (4376) reported further findings by themselves and coworkers about the metabolism of D_3:

1. Elaboration of 25-OH-D_3 in the liver was subject to feedback control. Time and dose relationships suggested that this control gave partial protection against D-toxicity, and that it conserved endogenous D for periods of low intake and low exposure to UV.

2. Animals fed low Ca diets metabolized 25-OH-D_3 mainly to 1,25-$(OH)_2$-D_3. Those fed normal or high Ca diets converted it mainly to 24-25-$(OH)_2$-D_3, suggesting that this might be the step at which animals adapted their D_3 metabolism to different levels of Ca intake.

3. To test this inference, chicks were fed low or high Ca diets, and were given either 25-OH-D_3 or 1,25-$(OH)_2$-D_3. Those given 25-OH-D_3 adapted their Ca absorption to low intake. Those given 1,25-$(OH)_2$-D_3 absorbed Ca at the same rate from both diets.

4. The enzyme system for 1-hydroxylation of 25-OH-D_3 was found only in kidney mitochondria, had a Michaelis constant of 2.7×10^{-6} M for intact mitochondria, was supported by O_2 and reduced cofactor, was inhibited by O_2-CO_2 mixtures and by the inhibitor DPPD, and was active in D-deficient tissues.

5. This system turned over rapidly. The enzyme had a half-life of 2.5 hours, and its mRNA about 6 hours. In the authors' view, the enzyme activity was not induced, but rather needed frequent transcription and new protein synthesis for its maintenance.

6. Chicks fed a strontium diet metabolized 25-OH-D_3 only to 24,25-$(OH)_2$-D_3, and the 1-hydroxylase enzyme was shown to be inhibited.

7. 1-Hydroxylation in isolated kidney mitochondria or in kidney slices was insensitive to additions of Ca.

8. Transfer of chicks from low Ca to high Ca diet elevated the serum Ca within 24 hours, but took days to switch the kidney enzyme system from 1-hydroxylation to 24-hydroxylation.

9. Removal of thyroids and parathyroids substituted 24-hydroxylation for 1-hydroxylation. This was reversed within 24 hours by 6-hourly administration of PTH.

10. However, since animals fed high Ca diet that was low in P 1-hydroxylated their 25-OH-D_3, PTH was not the only regulatory factor for this system. Further study was needed.

In 1974 Kenny et al. (3102) observed that ovulation in laying hens enhanced the metabolism of 25-OH-D_3 to 1,25-$(OH)_2$-D_3 relative to 24,25-$(OH)_2$-D_3.

In 1974 Hartenblower et al. (2477) observed in chicks depleted of D and given labeled D_3 and 1,25-$(OH)_2$-D_3, that these were taken up more by the liver and other tissues, suggesting that the liver was the principal site for

breakdown or further metabolism of 1,25-$(OH)_2$-D_3. However, in dogs similarly prepared, no such preferential disposition was observed, and the authors concluded that pathways were multiple.

In 1974 Zerwekh et al. (6355) confirmed that the synthetic analog 1α-OH-D_3 was as potent at stimulating Ca absorption in rachitic chicks as was 1,25-$(OH)_2$-D_3. Experiments with labeled 1α-OH-D_3 in vitro indicated that it probably was, in fact, converted to 1,25-$(OH)_2$-D_3 before expressing this activity.

In 1974 Baxter et al. (0409) observed in chicks that inhibition of Ca absorption by diphosphonates occurred at the 1-hydroxylation step.

In 1974 Jones and DeLuca (2974) found that chick kidneys metabolized 25-OH-D_3 to its 1,25- and 24,25-$(OH)_2$-D_3 metabolites as effectively as they metabolized the D_3 series, and concluded that discrimination by the chick against D_2 was not at the 1-hydroxylation step.

C. Excretion

In 1969 Bell (0465) studied the excretion of D_3 metabolites in bile by rats, using methods that circumvented the involvement of bile in the absorption of D_3.

He reported finding four chromatographic metabolites in the bile, and no trace of D_3 itself.

IV. Effects on Enzymes and Other Biochemical Parameters

Since traditionally the biochemical role of the vitamins D is to cure rickets, this Section begins with a statement of what rickets is -- or are, for many forms of it are now recognized. A convenient taxonomy was published in 1969 by Fraser (1900). His introductory definition was:

> "Any condition in the growing person in which there is a generalized failure of bone salts to be deposited promptly in the bone matrix and in the preosseous cartilage at the zone of provisional calcification."

Fraser classified such conditions in four main groups:

Group I - Rickets due to deficient intake or absorption of vitamin D, as detailed in Table 71.

Group II - Vitamin D-refractory rickets resulting from excessive renal loss of phosphate and other substances, as detailed in Tables 74 and 75.

Table 73

Features of the Three Stages of Vitamin D Deficiency (1900)

	Stage I	Stage II	Stage III
Onset Season	3 to 7 months January to April	5 to 12 months January to April	6 to 15+ months Any season, commonly spring
Mode of presentation	Generalized convulsions 75% or Tetany 25%	Classic skeletal signs or As incidental finding in acute respiratory or other infection	Classic skeletal signs Incidental finding in acute infection General "malnutrition" Convulsions or tetany
Additional physical signs	General physical status good Minimal or no skeletal signs of rickets EEG + or - (epileptiform)	Clinical rickets, moderate to severe Trousseau test[a] negative	Clinical rickets usually severe May have deformities Often malnourished, anemic, rarely scorbutic May have positive Trousseau test[a]
X-rays	Minimal signs of rickets	Classic skeletal signs, variable degree	Classic skeletal signs, moderate to severe
Serum Ca	+(9.0 to 5.0 mg per 100 ml)	Normal	+(9.0 to 5.0 mg per 100 ml)
Serum P_1	Normal	+(below 4.0 mg per 100 ml)	+(below 4.0 mg per 100 ml)
Serum Alk. P'tase	Slightly or moderately +	+ to +++	++ to +++
Urine	Normal amino acids	Generalized aminoaciduria	Generalized aminoaciduria

[a] Trousseau test: Obstruct circulation above elbow with B.P. cuff inflated above systolic pressure for 3 minutes. Watch for carpal spasm.

Table 74

Differential Diagnosis of the Four Commonest Types of Group II,

Hypophosphatemic Vitamin D-Refractory Rickets (1900)

Types	1. Hypophosphatemic vitamin D-refractory rickets (Simple type) (Familial vitamin D resistant rickets)	Fanconi Syndrome Group		
		2. Vitamin D-dependent rickets (Vitamin D-refractory rickets with aminoaciduria: Pseudo-vitamin D-deficiency rickets [Prader])	3. Cystine storage disease (cystinosis)	4. Tyrosyluria (tyrosinemia)
Onset	12 mos. to 18 mos.	3 mos. to 15 mos.	Infancy or preschool	Infancy or preschool
Genetics	Sex-linked dominant, or sporadic	(?)Autosomal dominant	Autosomal recessive	Autosomal recessive
Mode of presentation	Onset of bow-legs when starting to walk Short stature	Weakness, failure to stand or walk Generalized convulsions or tetany	*Infancy*: irritability, anorexia, failure to thrive, pallor, polydipsia *Preschool*: bow-legs, or knock-knees, short stature, pallor, photophobia	Failure to thrive, weakness, failure to stand or walk, irritability, anorexia
Additional physical signs	Healthy, stocky, strong Slight to moderate rickets No urinary symptoms	Severe, rapidly increasing rickets + deformities May have + Trousseau No urinary symptoms	Mild to severe rickets Usually pale, irritable, sickly Hair characteristically gray-blonde Polyuria (Occasionally hypothyroid)	Usually severe, rapidly increasing rickets + deformities Pathognomonic large, firm, modular liver ± hepatoma

Table 74 (cont.)

Types	1.	2.	3.	4.
Cystine deposits[a]	No cystine deposits	No cystine deposits	Cystine deposits, cornea and bone marrow	No cystine deposits
X-rays	Epiphyseal plate and metaphyses show mild to moderate rickets Shafts usually sturdy, well mineralized, coarse trabecular pattern = "chronic rickets"	Acute severe, active rickets Thin, poorly mineralized shafts Osteoporosis Pathologic fractures and pseudofractures	Rickets usually moderate, varies from mild to severe Osteoporosis common	Rickets varies from 0 to severe, active, with osteoporosis
Serum Ca	Always normal	Slight ↓ to marked ↓	Normal to marked ↓	Normal to moderate ↓
Serum P_t	Marked ↓	Moderate ↓ to marked ↓	*Early*: marked ↓ *Late*: normal to ↑	Marked ↓
Serum Alk. P'tase.	Slight ↑ to moderate ↑	Marked ↑	Moderate ↑	Marked ↑
Electrolytes and acid base	Normal	Normal	Normal or acidosis ± serum K ↓	Normal
BUN	Normal	Normal	Normal or marked ↑	Normal
Fasting blood sugar	Normal	Normal	Normal	Normal to ↓
Liver function tests	Normal	Normal	Normal	Abnormal
Plasma amino acids	Normal	Normal	Normal (cystine may be slightly ↑)	Tyrosine typically ↑ (± methionine ↑)
Urine				
Protein	0	0 → trace	+ to + + +	0 to + +
Glucose	0	0 → trace	+ to + + +	+ + to + + +
pH	Normal range	Normal range	Acid	Normal
Concentration	Normal range	Normal range	Normal to dilute	Normal to dilute
Urine aminoacids	Normal	Gross generalized aminoaciduria	Gross generalized aminoaciduria	Gross generalized aminoaciduria (especially tyrosine)

Table 74 (cont.)

Types	1.	2.	3.	4.
Prognosis	Life expectancy normal Usually remains hypophosphatemic, stunted and slightly deformed, despite therapy	Life expectancy normal "Cured" on high-dosage vitamin D therapy	Death from uremia at or before puberty	Death from cirrhosis or malignant hepatoma in infancy or childhood Dietary phenylalanine and tyrosine restriction may modify prognosis

[a]Cystine visible on cornea with standard ophthalmoscope (+40 lens) or with slit lamp. Visible on unstained bone marrow smears with Nichol prisms.

Table 75 (abstracted from 1900)

Syndromes not listed in Table 74	Physical signs	Blood	Urine
Oculocerebrorenal (Lowe's) syndrome	Onset: early infancy, Severe mental defect, Buphthalmos, Congenital cataracts, No deep tendon reflex, Almost always males	Low P	High amino acids (general)
	Onset of rickets at puberty	Glycine level normal	Glycine present in large amounts
HDRR* with hyperglycinuria	No special signs listed		
HDRR secondary to chronic lead poisoning, Wilson's disease, neurofibromatosis, etc.	No special signs listed		
Rickets secondary to renal tubular acidosis (Lightwood's or Albright's syndrome)	Failure to thrive, Kidney stones, Variable degree of rickets	Acidosis metabolic	Acid

*HDRR -- Hypophosphatemic vitamin D-refractory rickets

Group III - Renal osteodystrophy secondary to chronic renal insufficiency of various causes. Signs include:

1. clinical: anorexia, polydipsia, polyuria;
2. skeletal: late-appearing, age 2-3, see paper;
3. blood: azotemia, usually acidosis, high BUN, APase: P normal to high; Ca normal to low; low rbc count.
4. urine: high protein

Group IV - Rickets with normal homeostasis of serum Ca and P_1: primary matrix defect:

1. Metaphyseal dysostosis:
   a. skeletal: see paper;
   b. blood and urine: normal;
   c. genetics: autosomal recessive
2. Hypophosphatasia:
   a. onset in utero or in first 6 months:

b. skeletal: cranium obvious, see paper;

c. blood: low APase; Ca normal to high; normal P and other measurements;

d. urine: phosphorylethanolamine present; high P_i;

e. genetics: autosomal recessive.

As is documented in this monograph, physiologic exposures to UV radiation or physiologic intakes of the vitamins D cure or prevent Group I, while certain of the purified natural or synthetic "metabolites" of the vitamins D have been claimed to possess specific potential applications in some of the conditions listed in Groups II and III above.

A. Effects on Calcium and Phosphorus

In 1949 Migicovsky and Elmslie (3954) measured the Ca and P excretions of starving chicks. They found that D diminished Ca loss but had little effect on P loss, while Ca ingestion diminished P loss. When labeled Ca was fed, the authors found that the retained label was concentrated in bone.

In 1954 Pincus et al. (4565) studied five groups of infants less than a week old. Two groups were fed breast milk; one of these received D 600 USP units/day. Three groups were fed powdered milk formula; one received 400 USP units in the formula, another 600 as a vitamin supplement, and the third no D.

No breast milk fed infant had serum Ca less than 8 mg/100 ml. Of those fed formula, 10.9% of the D-free group had serum Ca less than 8 mg/100 ml, compared with 30% of those given 600 USP units of D, and these three groups did not differ from each other as to serum P, although the formula had 3-4 times the P content of the breast milk.

Under these conditions the authors concluded that D tended to lower serum Ca. A trend in the same direction was noted in the two groups fed breast milk.

In 1954 Carlsson et al. (0918) studied the influence of D (form not stated) on absorption of P_i by rats. When the rats were Ca-deficient, absorption of P_i was increased, and the authors concluded that the influence of D was indirect. When the Ca:P ratio was high, D had no influence on the absorption of P_i. However, in both cases D increased the absorption of Ca.

The authors concluded that the absorption of P_i was "fairly independent" of the absorption of Ca.

In 1955 Migicovsky and Jamieson (3956) found that when chicks were given Ca and D orally, the amount of absorption was dose-related to the fed Ca, and

that D allowed the chicks to adapt their capacity for absorption to different intakes of Ca. D had no effect on bone Ca when labeled Ca was given i.m.

In 1956 Conrad et al. (1125) gave lactating and non-lactating dairy cattle 30 million IU of D_2 by mouth daily for 7 days in capsules containing viosterol 1 million IU/g. Tracer doses of labeled Ca and P showed that the absorption of these minerals was increased several-fold, and their excretion was diminished. Rises of serum P preceded rises of serum Ca, and Ca label disappeared from the blood faster in lactating than in non-lactating cows. However, no effect of D was demonstrated on Ca label in milk.

In 1957 Conrad and Hansard (1124) reported similar findings in calves given 5 million IU daily for five days. Increased Ca levels in kidney and esophagus disappeared after eight days of D supplementation when two additional five day treatments were given. Femur section autoradiographs showed that deposited Ca became exchanged more rapidly.

The authors concluded that the rate of movement of Ca through the plasma was increased, because serum Ca levels remained "normal", and that the rate of tissue calcification was approximately tripled. When the rate of new bone growth was maximized the authors warned that calcification might occur in the soft tissues, although in their experiments this was transient.

In 1964 Thompson and DeLuca (5772) found that D_2 fed to rats tripled the incorporation of ^{32}P into gut mucosal phospholipids but not into nonlipid P compounds. Smaller, similar effects were seen in the kidney but not in the liver. The effects were not Ca-dependent, and the total phospholipids and rate of glycerol and serine incorporations into phospholipids of the gut were not altered. However, P_i incorporation was stimulated slightly, and was oxidation-dependent.

The authors concluded that this phospholipid effect of D might be primary and directly related to Ca transport (enough Ca may have been present in the tissues).

In 1967 Hunt et al. (2818) reported that D_3 but not D_2 promoted intestinal absorption of Ca^{47} in New World primates. Earlier studies had shown less or no antirachitic activity of D_2 vs. D_3 in several spp.: Cebus albifrons, Saguinis oedipus, S. nigricollis, S. mystax.

Six C. albifrons were fed D_2 2000 IU/kg of diet for 24 months and developed severe osteodystrophia fibrosa. D_3 was then substituted for D_2 for five months,

and the disorder vanished. D-free diet was then fed for 12 months, and the disorder returned. For 12 days two animals were given D_2 500 IU/day orally, two were given D_3, and two were untreated. Then the animals were fasted for 24 hours; each was then intubated with 7μC of Ca47 and fed 50 g diet, and the radioactivity of serum, urine, feces, and skull were determined at various time intervals. Further studies were conducted (see original paper for details).

By each of the criteria in the study, no difference was observed in Ca absorption between untreated and D_2 treated animals, and large differences were observed between these and the D_3 treated animals. When Ca^{47} was injected i.v., it disappeared from the serum at the same rate in all six animals, and most appeared in the feces of the untreated animals, less in those given D_2, and significantly least in those given D_3. Head radioactivity counts of those given D_3 were higher than of the other two groups, which did not differ from each other.

The authors concluded that D_3 but not D_2 promoted both the absorption and retention of Ca and its deposition in bone in these animals. They commented that these studies shed no light on any differences in mechanism of action between D_2 and D_3.

In 1968 Kowarski and Schachter (3275) studied the role of D_3 in the transport of phosphate across rat intestinal mucosa. They found that D_3 given to rats before death increased the transport of phosphate as measured afterwards in vitro.

In vivo experiments indicated that D_3 acted directly on the gut without prior activation. However, the transported $^{32}P_i$ mixed with only a small part of the P_i already in the mucosa, so that the effect of D_3 on its incorporation into metabolites could not be measured. This and other findings led the authors to conclude that the influence of D_3 on P transport was separate from its influence on Ca transport.

In 1969 Hashim and Clark (2491) used Ca^{45} to study the role of vitamin D_2 in Ca transport through the small intestine. Male weanling Holtzman rats were prepared by diet to be normal, hypo-, or hypervitaminotic D. Ca uptake and release were measured in vivo, and in vitro using mucosal cell suspensions mainly from tips of villi.

The authors reported that:

(1) Both hypo- and hypervitaminotic cells accumulated more Ca than normal cells.

(2) Cell uptake was found to be passive at 0°C, while at 38°C there seemed to be an active carrier system dependent on glycolytic energy.

(3) Release of Ca was depressed in hypovitaminotic suspensions.

(4) Release *in vivo* was delayed in D-deficiency and accelerated in D-excess.

The authors concluded that vitamin D was involved in the release of Ca from the mucosa into the blood, but not in its uptake from the intestinal lumen.

In 1969 Olson and DeLuca (4367) found that perfused intestine from D-deficient rats had about half-normal capacity to transport Ca. Addition of 2.5 µg 25-OH-D_3 normalized the Ca transport in two hours, while addition of D_3 to the perfusate had no effect during four hours.

In 1969 DeLuca (1453) summarized some of the work of his laboratory on the metabolites of D_2, D_3, and D_4.

He cited the finding of Stohs and DeLuca in 1967 that 25-OH-D_3 was accumulated by the nuclear membranes of the intestinal mucosal cells, where it induced transcription of mRNA specific for a Ca transport protein.

In 1971 Omdahl *et al.* (4375) reported on the biological activity of the new metabolite, 1 25-$(OH)_2$-D_3.

Chicks and rats were fed purified diets or in some cases the Steenbock diet (Table 1) plus predetermined amounts of Ca and D, and received labeled metabolites. Chick bioassays were performed using intestinal loops *in situ*. Rat antirachitic bioassays were performed according to the USP. Bone decalcification bioassays were performed on blood samples, by atomic absorption spectra. (See paper for details)

(1) The chicks responded to as little as 195 pmoles of 25-OH-D_3, peak responses occurred 24 hours after doses, and the dose-response curve was linear (Fig. 5). Responses to D_3 were about 5 hours slower. Responses to 1,25-$(OH)_2$-D_3 peaked at 10 hours and at twice the values of the responses to 25-$(OH)_2$-D_3. Two other metabolites elicited no responses. See Table 76 and Fig. 6.

(2) The rat antirachitic effect of 1,25-$(OH)_2$-D_3 was only 0.4 of the 25-OH-D_3 effect, and one of the two other metabolites had no effect.

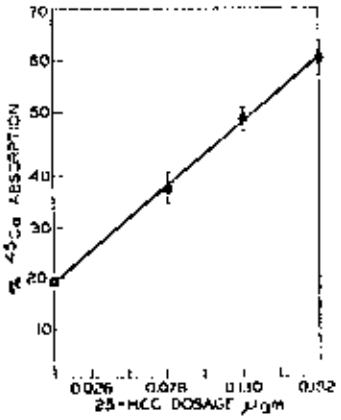


Fig. 5. Dose response curve for 25-HCC and calcium absorption in chicks. Chicks were injected in 24 hr prior to use. Each point represents the average of 6 chicks. The vertical bars represent standard error. (4375)

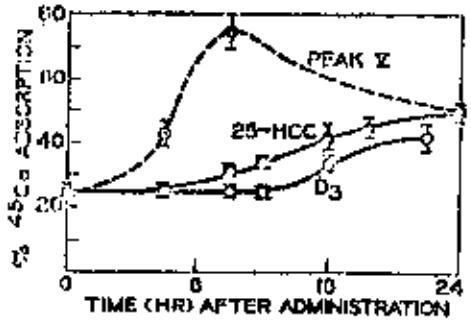


Fig. 6. Response of chick intestinal calcium absorption to 25-HCC, vitamin D_3, and peak V (1,25-DHCC). Each point represent the average of 4-7 chicks ± standard error. (O————O) 325 pmoles given iv; (O————O) 325 pmoles given iv; (O————O) 325 pmoles administered orally. (4375)

Table 76

Stimulation of Intestinal Calcium Absorption by 25-HCC, 1,25-DHCC, and Peak V Metabolites (4375)

Compound	Dosage (pmoles)	Dosage Method	ta (hr)[a]	RTAM (ta)/25-HCC$_{(t_{24})}$[b,c]
25-HCC	195-455	Iv and oral	24	1.00
Peak V_{t1}	227	Oral	24	0
Peak V_{t2}	258	Iv	24	0
1,25-DHCC	455	Iv	24	0.72
1,25-DHCC	455	Oral	24	1.09
1,25-DHCC	325	Oral	24	0.97
1,25-DHCC	325	Oral	10	2.00

[a] ta represents: time of assay following dosage. [b] (^{45}Ca absorption for metabolite at time ta)/(^{45}Ca absorption for 25-HCC at time 24 hr) where net ^{45}Ca absorption = ^{45}Ca absorption for metabolite − ^{45}Ca absorption for control. [c] RTAM = relative transport activity metabolite.

(3) The bone Ca mobilization response to 1,25-$(OH)_2$-D_3 was significant at 24 and 72 hours, responses to 25-OH-D_3 were significantly higher at both times, and both groups were back to normal at 96 hours.

The authors commented that they had obtained a bone Ca mobilization response to the 1,25 whereas Kodicek's laboratory had not.

They concluded that:

(1) If 1,25-$(OH)_2$-D_3 was the final active metabolite, made in the kidney, then it would be sequestered by the intestine, the target tissue. There, its action was quicker, briefer, and more intense than those of 25-OH and D_3.

(2) The total antirachitic effect of the 1,25-$(OH)_2$ was less than those of the longer-acting 25-OH and D_3.

(3) The data did not reveal which of the 25-OH and 1,25-$(OH)_2$ forms was the final active metabolite in bone decalcification.

In 1971 Boyle et al. (0705) gave 325 pmoles of 25-OH-D_3 to rats by intrajugular injection; 12 hours later 1,25-$(OH)_2$-D_3 was found to accumulate in the serum.

(1) When the Ca content of the diet was increased stepwise from 0.02% to 2%, so was the serum Ca content, while the 12-hour level of the 1,25-$(OH)_2$-D_3 diminished, and the level of another metabolite, 21,25-$(OH)_2$-D_3 increased.

(2) When the rats were injected with D_3 instead of 25-OH-D_3, these differences were accentuated.

(3) When the diet contained 3% Ca and 20% lactose, the formation of 1,25-$(OH)_2$-D_3 was suppressed.

The authors concluded that the production of the final active metabolite, 1,25-$(OH)_2$-D_3, was part of the mechanism by which rats adapted to low levels of dietary Ca.

In 1971 West (6178) commented on claims for therapeutic utility of 25-OH-D_3 made by DeLuca and coworkers. He pointed out that in D-refractory rickets little or no 25-OH-D_3 was elaborated endogenously when D_3 was given, but administration of the metabolite gave rapid, short-term responses. The author stated that this should enable the cumulative toxicity of D to be avoided in such cases, and the therapy to be finely adjusted to the patients.

In 1972 Glorieux et al. (2124) treated eight patients (aged 3-15) with X-linked hypophosphatemia for 1-8 years with oral P_i 1-4 g/day and D_2 10,000-50,000 IU/day.

On this regimen serum P averaged 4 mg/100 ml (85% of values over 3 mg), growth was accelerated, dwarfism was corrected in 5 cases, and X-ray evidence of rickets disappeared in all cases. Whole-blood oxygen pressure, low in untreated patients, was normalized, hypercalcemia was limited to five minor episodes, and no evidence of ectopic calcification was observed.

The authors commented that their combined P and D treatment involved inconveniences that were outweighed by its apparent clinical advantages, and that it provided "a useful way to neutralize the clinical effects of the mutation in X-linked hypophosphatemia, until a more basic approach is discovered to correct the defect in phosphate transport."

In 1973 Tanaka et al. (5684) reported that rats treated with 25-OH-D_3 had more active intestinal Ca transport when fed a low-P diet than when fed a normal-P diet. Also the same rats synthesized more 1,25-$(OH)_2$-D_3 in the kidney with the low-P diet.

The authors concluded:

(1) The low-P diet stimulated the 1,25-$(OH)_2$-D_3 production and, thereby, increased Ca transport.

(2) Since the hypercalcemia suppressed PTH secretion, PTH was not necessary for synthesis of 1,25-$(OH)_2$-D_3 by the kidney.

(3) However, kidney concentration of P_i could be "an important determinant" of 1,25-$(OH)_2$-D_3 production.

In 1973 Rasmussen and Bordier (4760) published an opinion on the natural history of osteogenesis at the cellular level in order to account for observations that were inconsistent with the classical Albrightian view of bone metabolism and turnover. Fig. 7 summarizes their preferred view, and Fig. 8 an alternative that they regard as "less likely." Fig. 9 summarizes hormonal influences, and the authors pointed out that in primary hyperparathyroidism doses of P_i often restored skeletal balance, while patients with renal osteodystrophy without evident D-deficiency had a secondary hyperparathyroidism and retention of P that resulted in positive skeletal balance. The authors claimed that their model explained why hypoparathyroid patients also showed a small net positive skeletal balance, due to stimulation of other areas of bone growth.

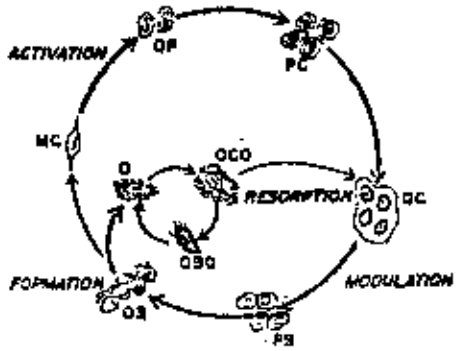


Fig. 7. Schematic Representation of the Sequence of Cellular Events in a Bone Remodeling Unit on the Endosteal Bone Surface. (4760)

The initial step (left) is the activation of mesenchymal cells (MC) to become osteoprogenitor cells (OP), which by further division become preosteoclasts (PC), which then fuse to become osteoclasts (OC). These eventually undergo modulation to become preosteoblasts (PB), which go on to become osteoblasts (OB), which after completing their synthetic function become osteocytes(O). Once the sequence of events has transpired, the osteocytes in a bone metabolic unit function in maintaining mineral homeostasis. In carrying out this function, they recapitulate, in a sense, a similar cell cycle of resorption (osteoclastic osteocyte - OCO) and formation (osteoblastic osteocyte - OBO). In severe hyperparathyroidism, the osteoclastic phase of the osteocytic cell cycle may be exaggerated to the point where several adjacent osteocytes remove all the bone between them, and then fuse to become osteoclasts.

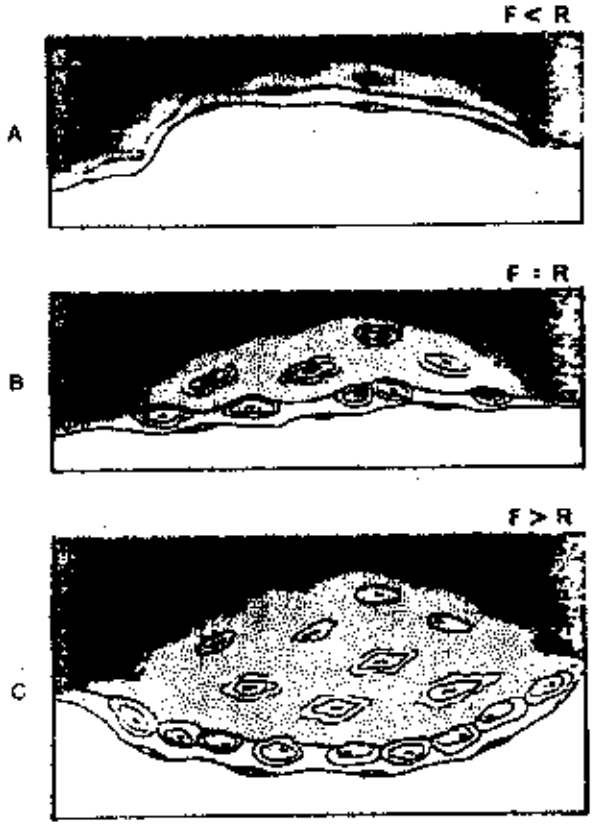
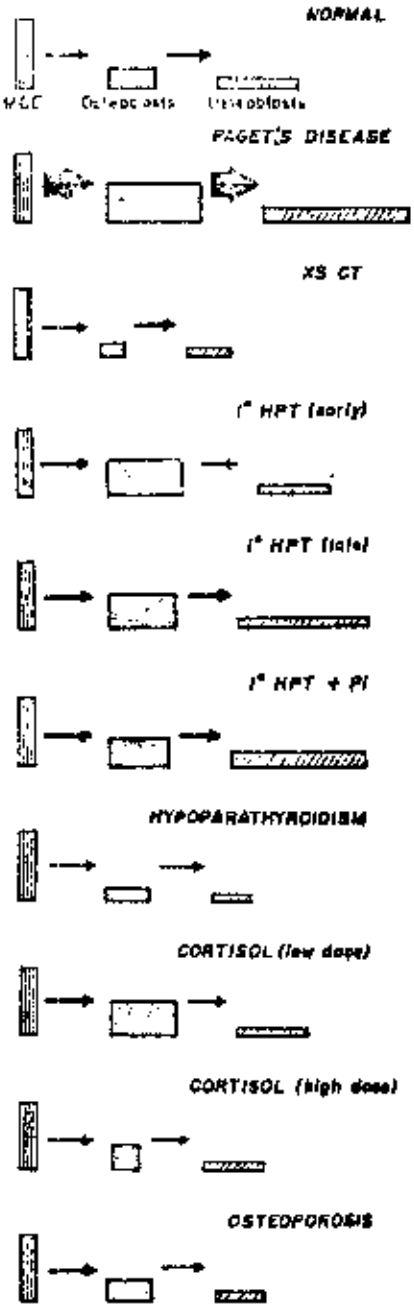


Fig. 8. The Three Possible Results of Remodeling Events in a Single Bone Remodeling Unit. (4760)

The formation (F) phase may not replace the bone during the resorption (R) phase (A); the two may be balanced (B), or the formation phase may add more bone than that removed during the resorption phase (C).



Height of blocks represents the extent of cell activity, and width the size of the respective cell pool. Thickness of arrows represents the rate of flow of cells from one pool to the next. In the normal young adult, the rate of flow of cells from cell in the mesenchymal cell envelope (MCE) to osteoclasts and the subsequent flow of osteoclasts to osteoblasts are equal, so that the total activities of the two pools are equal and skeletal homeostasis is maintained. The consequences of the various hormone and pathologic states are discussed in the text. 1°HPT indicates primary hyperparathyroidism, XS CT, excess circulating calcitonin, and PI, inorganic phosphate.

Fig. 9. Changes in Cell Activity and in the Size of Osteoclast and Osteoblast Pools in Various Metabolic Bone Diseases or Hormonal States in Man. (4760)

In 1973 Omdahl and DeLuca (4376) reported further metabolic effects of the D_3 compounds:

(1) At physiologic doses 1,25-$(OH)_2$-D_3 or perhaps a further metabolite of it was the agent of Ca mobilization from bone. However, at high overdosage 25-OH-D_3 was more active than 1,25-$(OH)_2$-D_3.

(2) However, in vitro (tissue cultures), 13,000 pmoles/ml of D_3 did not mobilize Ca from bone, 65 pmoles of 25-OH-D_3 was active, and 65 pmoles of 1,25-$(OH)_2$-D_3 was 100 times more active than the 25-OH-D_3.

(3) For transport of Ca across isolated rat intestines in vitro, 25-OH-D_3 was active at 60,000 pmoles/intestine; 1,25-$(OH)_2$-D_3 was more rapidly active at 65-325 pmoles, and was not further metabolized.

(4) 1,25-$(OH)_2$-D_3 had hardly any antirachitic activity when given seven days before a USP line test, or when given orally in oil at the plasma Ca of rats fed low Ca diet, while similar doses of 25-OH-D_3 did maintain the plasma Ca.

(5) However, 1,25-$(OH)_2$-D_3 given i.v. was as effective as 25-OH-D_3 at maintaining serum Ca, and was 2-5 times more potent at supporting bone calcification and at maintaining serum P level in rats fed low P diet. The authors concluded that 1,25-$(OH)_2$-D_3 turned over rapidly, and should be given by injection.

Reviewing these and other results to date, the authors (4376) summarized the principal actions of D as follows:

(1) Required for normal calcification of bone.

(2) Required for homeostasis of plasma Ca levels sufficient for normal calcification of bone.

These actions involved three principal mechanisms, outlined in Fig. 10:

(1) Absorption of Ca and perhaps also P from the gut.

(2) Mobilization of Ca from bone into the plasma.

(3) Reabsorption of Ca and P by kidney tubules.

These actions were properties, not of D_3 which was inactive, but of its metabolites, and especially of:

(1) 25-OH-D_3 — possibly active in Ca mobilization from bone; inactive in Ca absorption from the gut.

(2) 1,25-$(OH)_2$-D_3 -- direct activator of Ca absorption.

(3) 24,25-$(OH)_2$-D_3 -- abundant in tissues; active in Ca mobilization from bone; slightly active in Ca absorption from gut.

(4) 25,26-$(OH)_2$-D_3 -- active in Ca absorption from gut; almost inactive in Ca mobilization from bone and in bone calcification.

The authors suggested that metabolites (3) and (4) might be intermediates in the breakdown and excretion of metabolites (1) and (2).

D_2 was as effective in rat and man as D_3. It was known to be converted to 25-OH-D_2, but further studies were lacking. In chicks 25-OH-D_2 was almost inactive, and the possibility that its onward metabolism and excretion were accelerated required study (4376).

The principal mechanisms were further discussed in some detail (4376):

(1) Ca absorption involved a specific carrier protein (perhaps more than one), and also structural changes in the brush-border membrane. See Fig. 11.

(2) The rate of Ca absorption depended on the flow rate of 1,25-$(OH)_2$-D_3 from the kidneys, where 1-hydroxylation of 25-OH-D_3 was normally regulated by the supply of PTH. Thus PTH acted as tropic hormone for the D_3-derived hormone (see Fig. 12).

(3) Ca mobilization from bone was a concerted effect, in the bone, of PTH and either 25-OH-D_3 or one of its metabolites. Mobilization was inhibited by calcitonin independently of the D_3 metabolites. How this system worked was still to be discovered.

(4) Ca deposition in bone resulted simply from supersaturation of the plasma with Ca x P, resulting from absorption and mobilization. Thus Ca moved continually between plasma and bone.

(5) The D metabolites <u>possibly</u> enhanced absorption of P from the gut, and <u>possibly</u> enhanced reabsorption of Ca and P by kidney tubules.

(6) 1,25-$(OH)_2$-D_3 acted faster than its 25-OH-D_3 precursor, which in turn acted faster than D_3. Not only faster, but also more briefly.

Some effects on these mechanisms were:

(1) 1,25-$(OH)_2$-D_3 had little and transient antirachitic activity, by bioassay, after oral ingestion; it might be more effective by injection.

(2) However, 1,25-$(OH)_2$-D_3 was effective when given to rats without kidneys; it by passed both sites of control, whereas exogenous 25-OH-D_3 bypassed only the feedback controls in the liver.

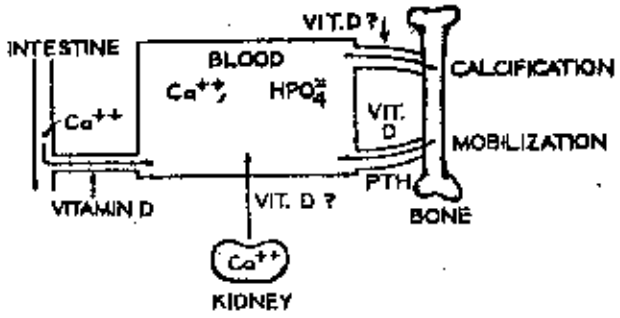


Fig. 10. Schematic summary of physiological actions of vitamin D.(4376)

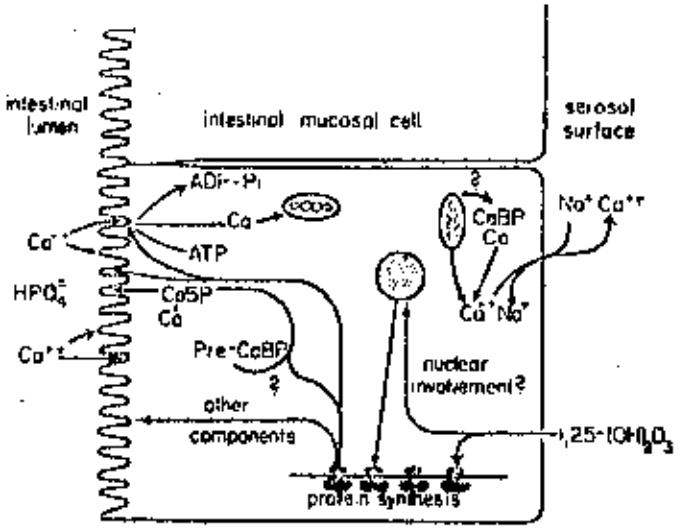


Fig. 11. Schematic summary of possible function(s) of 1,25-$(OH)_2D_3$ in calcium translocation in the intestinal mucosa cell. (4376)

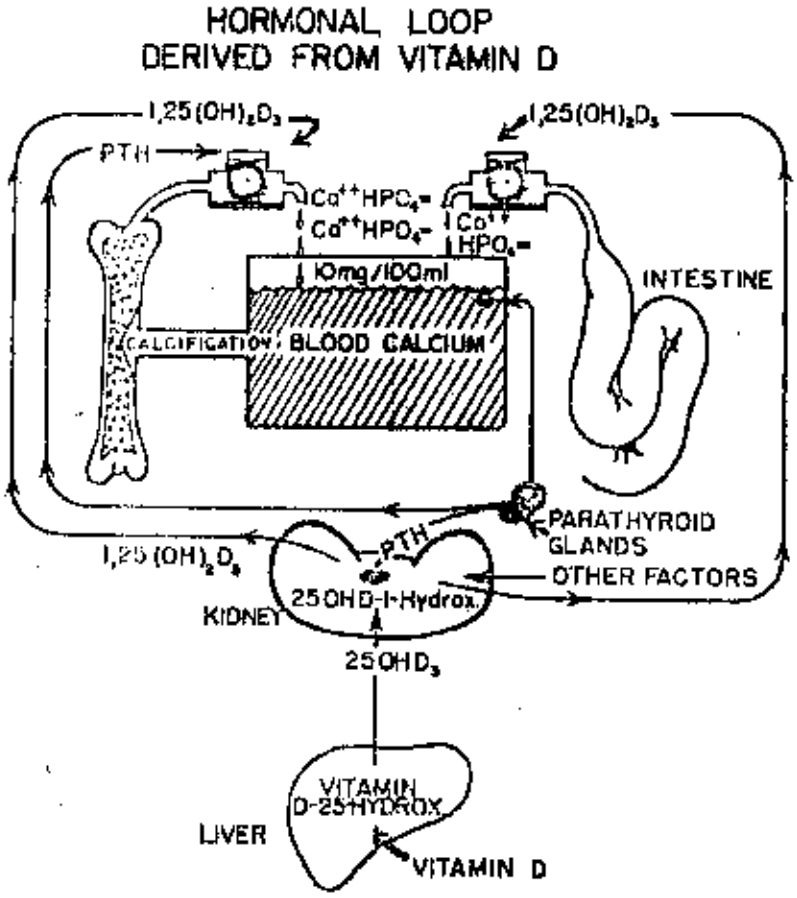


Fig. 12. Schematic designation of hormonal control loop for vitamin D metabolism and function. A drop below set point for serum calcium of 10 mg/100 ml prompts a proportional secretion of parathyroid hormone that acts to increase bone resorption and thus elevates serum calcium. Parathyroid hormone also directs metabolism of 25-OHD$_3$ to 1,25-(OH)$_2$-D$_3$ in kidney, where "hormone" 1,25-(OH)$_2$-D$_3$ acts both on bone and intestine to mobilize calcium from bone and intestinal contents. As serum calcium increases toward its set point, parathyroid hormone secretion is proportionately decreased. (4376)

(3) Several drugs affected these mechanisms in various ways.

Some special effects of synthetic analogs of D_3 on these mechanisms included:

(1) The dihydrotachysterols had 1/450 of the antirachitic potency of D_3 in physiologic doses, but high doses were markedly effective. Dihydrotachysterol$_3$, synthesized by the authors, was more effective than D_3 in high doses, and was effective in rats without kidneys.

(2) 5,6-*trans*-25-OH-D_3, synthesized by the authors, was as effective as 25-OH-dihydrotachysterol$_3$, also synthesized by the authors, at stimulating Ca absorption in rats without kidneys, but did not enhance Ca mobilization from bone.

(3) 5,6-*trans*-D_3, synthesized by the authors, was effective on both systems in rats without kidneys.

(4) 1α-OH-D_3, synthesized by the authors, was effective on both systems and also on bone calcification. Given by mouth, it had more antirachitic potency than the natural D_3-derived hormone, and it bypassed the kidney step. Whether it required the addition of 25-OH by the liver was uncertain. (Later work by others (6355) has indicated that 25-hydroxylation does occur).

The authors gave three reasons for studying the D_3 metabolites and synthetic analogs:

(1) To elucidate the modes of action of the vitamins D.

(2) To reveal the etiologies of the D-resistant forms of rickets and other disorders of Ca and P metabolism.

(3) To develop specific, low-dose treatments of these disorders that would eliminate the hazards of current treatments with massive doses of D vitamins.

B. Effects on Calcium-binding Protein (CaBP)

In 1965 Norman (4303) found that the Ca-absorption responses of rachitic chicks to D_3 were actinomycin sensitive, and concluded that the responses required an unimpaired RNA-synthesizing system.

In 1965 Taylor and Wasserman (cited in 1557) discovered calcium-binding protein (CaBP) in chicks.

In 1967 Kodicek (3214) found that the transport of Ca across rat intestinal mucosa was active and at least partly D-dependent; there was no D-dependence at high Ca concentrations (125 mM) in the lumen. Other experiments suggested stimulation of transport by parathyroid hormone (PTH).

The author commented that in his studies no evidence was found for a suggested involvement of phospholipids in D-dependent Ca transport. However, his data would be consistent with other suggestions cited, of DNA- or RNA-linked synthesis of a CaBP (3214).

In 1969 Avioli et al. (0269) reported that uremic rats had less CaBP activity in duodenal mucosa than normal rats, and oral D_3 made no difference. However, prior doses of 25-OH-D_3 increased the CaBP activity and the transport of Ca^{45}.

The authors suggested that:

(1) 25-OH-D_3 directly stimulated transcription, or indirectly altered nuclear membrane permeability, in the mucosal cells,

(2) the resultant protein was either a translocase or transport enzyme, or was identical to the chick mucosal D-dependent CaBP described by Wasserman (cited), and

(3) if so, any decrease of 25-OH-D_3 in the gut in uremia could diminish synthesis and concentration of CaBP and so could account for the characteristic Ca absorption defect of uremia.

In 1971 Tanaka et al. (5687) reported that the Ca transport response to 25-OH-D_3 was blocked by actinomycin D but the response to 1,25-$(OH)_2$-D_3 was not. From this they concluded:

(1) that the 1,25-$(OH)_2$-D_3, or a further metabolite of it, and not 25-OH-D_3, was the form of D_3 responsible for effects on Ca transport in the intestine,

(2) that the effects did not involve transcription of DNA, and

(3) that all effects of D_3 on Ca transport in the intestine were dependent

on metabolism of 25 OH- to 1,25-$(OH)_2$-D_3 in the kidney, which did involve transcription of DNA.

In 1971 Drescher and DeLuca (1557) reported that they had purified rat CaBP.

In review they noted that CaBP was first observed in the intestines of chicks with rickets by Taylor and Wasserman (cited as Nature 205: 248, 1965), and later was reported in rats, dogs, calves and monkeys by various authors (cited). It appeared in the mucosa in response to D. Estimates of mw varied in the literature for example, 13,000 by gel filtration, 25,000 by sedimentation equilibrium, and two proteins of 24,000 and 145,000 by different techniques.

The authors reported that a combination of column chromatography and gel filtration had revealed two homogeneous proteins, one possibly being the precursor of the other. The final CaBP was confirmed as homogeneous by column chromatography, disc gel electrophoresis, and ultracentrifugation. However, the mw was 8,000-9,000 by sedimentation, while it was 13,000 by gel filtration and by electrophoresis.

The authors attributed the discrepancy to one of:

(1) Asymmetry of the molecule (preferred, and if so, the smaller mw would be correct), or

(2) Specific volume augmented by, e.g., bound lipid (if so, the larger mw would be correct, but considered less likely).

In 1973 Chapman et al. (0973) reared some guinea-pigs in the dark for 16 weeks, giving them nil to 220 IU D_3 per 100 g diet. They found that with a normal Ca:P ratio in the diet the guinea-pig did not need exogenous D to prevent rickets during most of its growth, and that its intestinal CaBP was not D-dependent; if exogenous D was needed, then the amounts needed were too small to be bioassayed in the diet.

C. Parathyroid and D Interaction

In this monograph the expression PTH means parathyroid hormone, and is used for studies that were in fact carried out using parathyroid extract, as all authors cited make clear. Since it was early concluded that the effects cited were those of the hormone and not of a contaminant (3154), and no contrary opinions were found, the single expression PTH has been adopted for clarity.

In 1953 Klein and Gow (3154) studied children aged two weeks to 12 years who had no evidence of kidney dysfunction but various other disorders. They

studied the responses of the kidneys to injections of PTH. They found that PTH increased the glomerular filtration rate and diminished reabsorption of P. On the other hand D increased the filtration but did not inhibit the effect of PTH. The authors inferred that D inhibited the secretion of PTH.

In 1964 DeLuca and Sallis (1456) reported on some subcellular actions of PTH in relation to D, found during in vitro experiments.

As in kidney tubules, so in mitochondria PTH increased Ca efflux and P_i influx. So did D, perhaps synergistically with PTH, and perhaps through a metabolite of D.

PTH depressed oxidative phosphorylation but unexpectedly in some cases stimulated respiration, suggesting an increase of non-phosphorylative oxidation. This respiration required substrate and also both P_i and Mg^{++}. It was shown that PTH stimulated uptake of P_i, and that Mg^{++} was transported along with P_i. Less effectively, PTH could be made to transport $HAsO_4^{--}$ or SO_4^{--}, also with increased respiration. However, although other anions could be substituted for P_i, the authors had been unable to substitute other cations for Mg^{++}.

The involvement of PTH in mitochondrial respiration was supported by results of PTH-dependent ATPase studies.

Nevertheless the authors cautioned that these experiments had involved high doses of PTH and, where D was involved, extreme degrees of deficiency before replacement. They concluded that mitochondria showed promise as a system in which to study the biochemistry of D and PTH relationships.

In 1965 Nay et al. (4236) measured clearances of Ca, P, and hydroxyproline in rachitic dogs before and after removal of their parathyroids and after PTH replacement therapy. They reported that:

(1) Before removal, serum P was lower, and P clearance was higher, in rachitic dogs than in controls.

(2) After removal, urinary P and P clearance decreased, showing that the tubules could could conserve P despite D deficiency, and that endogenous PTH could decrease P conservation at the tubules despite D deficiency.

(3) After removal serum Ca fell, and it rose with subsequent PTH replacement. This could not be due to changes in urinary Ca, and suggested an action of PTH on bone.

(4) Urinary hydroxyproline rose after removal, and fell after replacement of PTH, suggesting an action of PTH on collagen metabolism during D-deficiency.

In 1966 Harrison (2448) reviewed work in his and other laboratories on the relationship between PTH and D. He concluded that D was needed for the full expression of PTH activity. He surmised that the effects of D on cell permeability to Ca might explain findings that D could facilitate or substitute for PTH.

In 1966 Arnaud et al. (0230) found that responses of parathyroidectomized rats to PTH infusions differed according to whether the rats were fed or deprived of D. Measurements included plasma Ca and P, and urinary Ca, P, Mg, Na, and K.

They concluded that in the presence of physiological concentrations of PTH, D was necessary to mobilize Ca and P from bone but not for the action of PTH on the kidney tubule.

In 1967 Marnay-Gulat (3719) reported that in parathyroidectomized rats, guinea-pigs, and chicks, the influence of purified PTH on blood Ca level was dependent on small amounts of exogenous D. On the other hand, the PTH effects on serum and urinary P were not always D-dependent.

In 1968 Grose and Scriver (2269) studied the relationships between PTH and urinary excretion of P and amino acids in rats made hypocalcemic by Ca- and/or D-deprivations.

(1) The excretions became excessive when blood Ca fell below 6.5 mg/100 ml, and the P excretion was excessive 2-4 weeks before the amino acid excretion.

(2) Ca injections that raised blood Ca above 6.5 mg/100 ml normalized the excretions within six hours.

(3) PTH increased the excretions even when it also raised the blood Ca.

(4) The size of the parathyroids increased in proportion to the blood Ca deficit and the excretion excesses.

(5) Removal of the parathyroids normalized the excretions within six hours.

The authors concluded that the effects of D-deficiency on excretion of P and free amino acids occurred in the following order:

D deficiency,
Diminished Ca absorption and Ca mobilization from bone hypocalcemia,
Hypocalcemia,
Secondary hyperparathyroidism, and
Inhibited tubular absorption of P and amino acids.

In 1968 May et al. (4235) showed that D_3 had activities independent of PTH. Thirteen mongrel pups were caged, deprived of UV, and became rachitic clinically and biochemically in 3 months. Then, thyroids and parathyroids were removed, and each animal was given thyroid extract 65 mg/day. Clearance studies were conducted under control conditions and after various treatments with D_2, D_3, Ca, and PTH.

The authors found that:

(1) D decreased tubular reabsorption of P, and this "often" was seen before the D-induced rise of serum Ca.

(2) Effects of D similar to those of PTH were "much slower in onset", and this could not be attributed to delayed absorption of the D.

(3) Unlike PTH, D did not increase the urinary output of hydroxyproline.

The authors commented that large doses of D were needed to maintain hypoparathyroid dogs, as with humans, and that smaller doses used in the presence of intact parathyroids "might not have demonstrable tubular effects."

In 1969 Trummel et al. (5847) studied in vitro the effects of small concentrations of 25-OH-D_3 on mobilization of previously incorporated Ca^{45} from rat bones in tissue culture. Earlier, large doses (300-400 IU/ml) had given inconsistent results, and the doses used here, 0.9-27 IU/ml, constantly released the Ca^{45}. The time course was similar to that of release produced by PTH, and both were inhibited by calcitonin. When PTH and 25-OH-D_3 were added together, more Ca^{45} was released than the sum of separate additions, and the authors inferred synergism.

They noted that the low-level additions were comparable with concentrations resulting from therapeutic doses of D_3 in vivo, and suggested trial of 25-OH-D_3 as a superior therapy for hypocalcemia.

In 1970 Suh et al. (5593) reported that bone Ca was mobilized by PTH in a female with pseudohypoparathyroidism and receiving 100,000 IU/day of D_2. The response was seen when Ca intake was low, but was minimal in the absence of D_2 therapy.

In 1971 Arnaud et al. (0228) reported studies on patients with X-linked dominant hypophosphatemia in which males, but not always females, had rickets. Part of this disorder was impaired kidney tubular reabsorption of P, and the excessive urinary excretion of P could be abolished by i.v. infusions of Ca.

The question was whether the disorder was caused by a defect in the metabolism of D, or by a primary defect in P transport. In the first alternative PTH secretion should be excessive, in the second it should be normal.

The authors found that serum immunoreactive PTH was normal in their patients, did not influence tubular reabsorption of P in the males, and was increased by administration of phosphates. They concluded that these patients contained a P transport system that was insensitive to PTH but, in the kidney, was responsive to Ca.

In 1972 the same authors reported (0227) that the greater the D-deficiency, the higher were the serum PTH levels.

In 1972 Glorieux and Scriver (2122) investigated 15 patients with X-linked dominant hypophosphatemia, described as D-resistant rickets in which over-excretion of P_i by the kidneys responds to i.v. infusions of Ca.

The authors hypothesized that the kidney anomaly was primary, rather than secondary to impaired D metabolism and Ca absorption with excess PTH production, because the condition also responded to P_i replacement.

Using various methods (see original paper) they observed:

(1) That a PTH-sensitive factor for P_i transport was responsible for about two-thirds of P_i reabsorption in normal human kidneys.

(2) This factor was partly absent in females with X-linked hypophosphatemia and totally absent in male patients.

(3) Male patients had a residual factor that could be saturated, as in normals, that permitted two-way flow of P_i across the tubules, and that was not sensitive to PTH but was sensitive to Ca^{++}.

The authors stated that not all features of the condition could be explained by these occurrences in the kidney, and suggested that P_i transport across other cell membranes might be impaired in ways corresponding to the (probably different) systems for P_i transport in other membranes. In addition they suggested that different impairments of P_i transport would be found to described the different clinical variants of X-linked hypophosphatemia.

In 1973 Kind et al. (3136) reported in an abstract that D enhanced the effects of PTH on renal handling of P and on mobilization of Ca from bone, both in hypo- and in pseudohypo-parathyroidism. At the same time there were no effects on changes of urinary cAMP levels. The authors commented that this showed that the renal handling of P was "not entirely mediated" by cAMP.

D. Effects on Citrate Metabolism

In 1953 Steenbock and Bellin (5521) found in rats that physiological doses of D increased the citrate content of blood, bone, kidney, heart, and small gut but not of liver. Presence or absence of bicarbonate was irrelevant. The authors concluded that observed increases in urinary citrate reflected these accumulations.

In 1954 Bellin et al. (0468) found that D increased the excretion of urinary citrate by rats fed diets of widely differing mineral contents. Although high P diets resulted in higher citrate excretion than low P diets, potassium phosphate supplements diminished citrate excretion. The pH was found irrelevant, and additions of $NaHCO_3$ had little effect. The authors concluded that D acted to increase citrate synthesis rather than to decrease its destruction.

In 1954 Tulpule and Patwardhan (5859) studied Embden-Meyerhof pathway (EMP) activity and pyruvate oxidation in cartilage of rachitic and D-treated rats. They found no shifts of EMP activity but pyruvate oxidation was diminished in rickets and restored by D. They inferred that this might influence the formation of citrate, for which a role in bone calcification had been proposed.

In 1961 Rao and Patwardhan (4725) studied the glycolytic-lipogenic pathway enzymes in rat cartilage during experimental rickets and its healing by D_2. The formation of acetyl lipoate and CoA were diminished and increased respectively, and the authors concluded that this could account for reports of similar effects on citrogenase activity.

In 1962 Sahashi et.al. (4991) found that D_3 was more antirachitic than D_2 in chicks, judged by analyses of bones for P, Ca, and total ash. D_3 also increased citrate and pyruvate levels in blood and kidneys. Sulfate-S^{35} was also markedly increased in tibias after D_3.

In 1961 Rao and Patwardhan (4725) investigated the role of citrate metabolism in D-deficiency rickets.

A total of 144 young rats were made rachitic by a low-P diet and some were given D supplements. Cartilage was then incubated for in vitro studies.

The authors found that formation of acetyl lipoate from ATP and acetate in the cartilage was diminished by P deficiency and was restored during healing induced by D. The restoration was significant at 48 hours after D, and at 96

hours there was no further significant increase.

The authors inferred that losses of enzyme activities started with aceto-CoA-kinase (forming acetyl-CoA from acetate and CoA in the presence of ATP); and thence ATPase (pyrophosphatase) and citrogenase would also decrease (as reported by others cited), since citrate appeared to be an important intermediate in the relevant pathway.

In 1961 Bhussry and Kiguel (0539) inferred from histological and radiographic evidence in newborn rats fed the Steenbock rachitogenic diet, that the effects of D on bone were expressed in the matrix-producing cells, and probably reflected alterations of citrate accumulation.

In 1963 Guroff et al. (2318) disputed the likelihood that citrate was concerned in the effects of D on bone. They found:

(1) that D-induced rises of serum citrate were diminished by deficiencies of pantothenate and pyridoxine in rats without affecting the D-induced increases of serum Ca or P, or bone ash.

(2) Pyridoxin deficiency diminished the response of bone citrate to D.

(3) With low-Ca diet, cortisone prevented the serum and bone citrate responses to D, without affecting the serum Ca responses.

E. Effects on Amino Acids

In 1961 Engstrom et al. (1687) determined the levels of various amino acids as well as free α-amino N in the urine of rats fed diets with and without D_2. Unlike human infants with rickets, no aminoaciduria was found in the D-deficient rats at any stage of deficiency.

In 1965 Harrill and Gifford (2419) fed controlled amounts of D_2 for six weeks to weanling male rats and found that D_2 increased the plasma levels of free lysine but not of methionine or valine. D_2 also increased liver cholesterol and total lipids when protein was low (casein 9%); but these lipids decreased, and the effect of D_2 vanished, when casein 18% was fed.

The authors concluded that the amino acid:D_2 ratio might influence lipid metabolism.

F. Effects on Phosphomonoesterases

In 1955 Fraser et al. (1895) briefly announced findings of a ninhydrin-positive substance in the urine and plasma of a patient with "hypophosphatasia", or rickets with low serum APase. The abnormal substance was also found in the urine of the patient's healthy father, supporting the theory that hypophosphatasia was hereditary.

In 1957 Fraser (1899) reviewed hypophosphatasia and described 35 more cases. In some, cortisone therapy had been successful, but in others not.

The author concluded that the low serum APase activity reflected low enzyme concentration, owing possibly to increased degradation but more likely to decreased synthesis. He suggested that the root cause of the disorder lay in the bone, in some inhibitor of mineralization, that hypersensitivity to D might be a factor, and that the serum was unlikely to contain the cause of the disorder.

In 1969 Scriver and Cameron (5202) reported a condition that they called "pseudohypophosphatasia" because all the features of hypophosphatasia were present except low serum APase. Also, the APase was electrophoretically similar to APase from normal subjects. They stated that the primary abnormality "is yet to be found."

In 1967 Kiguel (3128) found less APase activity in developing molars of D-deficient rats than in controls or D-supplemented rats. Even less APase activity was found in D-deficient rats fed diets with elevated Ca:P ratios. Parallel differences of mucopolysaccharide and mineral content suggested to the author that APase might influence mucopolysaccharide synthesis. He commented that the apparent influence of D on APase activity was not explained; also, that while humans became rickety from D-deficiency alone, distortion of the Ca:P ratio was also required for rats to show rickets. In rats, according to the author, decrease of APase activity preceded clinical rickets.

In 1969 Martin and DeLuca (3736) studied the need for alkaline phosphatase (APase) during normal osteogenesis in chicks. Although the involvement of APase in cell division was a matter of general knowledge, induction or increase of activity had been reported under some conditions of bone tissue culture and not others.

They found that activity was stimulated by a factor which they could part-describe as a nonprotein molecule that was negatively charged, heat-stable, base-stable, acid-labile, slowly dialyzable, charcoal-adsorbable, and not hydrolyzed by bacterial phosphomonoesterase or low-specificity proteinase. They suggested that this molecule was an APase inducer.

During these studies they eliminated, as potential inducers, L-cysteine, proline, OH-proline, hexosamine, aminoacids nonessential to their cultures, inorganic pyrophosphate, and ascorbic acid.

In 1969 Scriver and Cameron (5202) described a single human three-month female infant with classic hypophatasia except that total alkaline phosphatase activity in her plasma was consistently normal. However, the APase activity with low concentrations of P-ethanolamine was subnormal, and P-ethanolamine levels in the patient were elevated.

The authors surmised a rare allele, and proposed to name the condition "pseudohypophosphatasia."

In 1970 Hausaler et al. (2510) investigated the site and mechanism of intestinal APase induction by D_3.

1. They produced increases of Ca absorption and actinomycin-sensitive APase activity by giving 50 IU to rachitic chicks.

2. Then they found that the APase activity in isolated microvilli was not abolished by Triton X-100, showing that the increase was a property of the enzyme itself.

3. In the same preparation they also found increases of Mg^{++}- and Ca^{++}-activated ATPase but not of invertase, showing that the increases were not due to proliferation of microvilli.

4. Butanol extractions released and augmented all three enzyme activities, and other reactions indicated that all three were properties of a single enzyme protein.

The authors concluded that the microvilli were sites of D_3 action, and that the changes of phosphatase activity were related to Ca absorption.

G. Effects on Clinical Hypercalcemia

In 1958 Fellers and Schwartz (1773) studied three infants with idiopathic hypercalcemia and four normal infants. The three were reportedly not the common and mild Lightwood type cases but the severe type in which Fanconi had listed growth retardation with eventual dwarfism, mental deficiency, osteosclerosis, and kidney malfunction with aminoacidemia (azotemia) and high Ca levels in blood and urine.

The four normal infants had serum D activities of 100-200 IU/100 ml. The three hypercalcemics had serum D activities of 1700-1600 IU/100 ml comparable to another infant with D-resistant rickets receiving oral D 62,500 IU/day. However, the three had received only 1500 IU/day before diagnosis, and this did not account for their serum D levels 14 months after withdrawal of the supplement. The data (see paper itself) showed altered Ca and P metabolism.

The authors commented that Jeans and Stearns (2933) had reported growth retardation with doses of more than 1800 IU/day. They queried whether their findings could be accounted for by kidney tubular malfunction. They suggested the presence of another sterol, not D_3, with D activity; their lipoprotein values would be consistent with defective transport of such a sterol, and the hypocalcemia in their three cases was severe enough to be related to some defect in the metabolism of such a sterol.

In 1964 Garcia et al. (2025) drew attention to the "elfin facies" of idiopathic hypercalcemia, that it was similar to that seen in supravalvular aortic stenosis. They reported one case of a nine-month old boy with both disorders, claiming this as the "first proved association" of the two.

They suggested that idiopathic hypercalcemia was part of the mental abnormality syndrome with supravalvular aortic stenosis and elfin facies, emphasizing the similarity of the cardiovascular lesions in idiopathic hypercalcemia and in experimental D intoxication.

In 1966 Taussig (5703) reviewed idiopathic hypercalcemia of infants and drew attention to aspects that had been mentioned but not emphasized by predecessors including Bongiovanni et al. (0656)

He stated that the hypersensitivity was equivalent to an abnormally efficient response to D, could be classed as "an inborn error of metabolism," and could arise from metabolism of the vitamin itself.

Such infants, in the author's view, were injured by 3000 IU/day but not by 400 IU/day, the safety margin being only 8-fold.

In 1967 Fraser et al. (1897) studied 39 infants with "simple" D-deficiency, and described three progressive stages:

Stage I: hypocalcemia and convulsions.

Stage II: rickets; blood Ca normal but low P; high urinary P and amino acids.

Stage III: Stage II plus return of Stage I.

PTH aggravated the progress of Stages I-III. On the other hand, infusions of D and Ca raised the blood Ca and removed the urinary excesses of P and amino acids.

The authors concluded:

1. The urine data reflected PTH effects on kidney tubular reabsorption.
2. Excess PTH resulted from low serum Ca.

3. Low serum Ca resulted from D-deficiency, because intestinal transport of Ca was not stimulated.

4. Nevertheless, D-deficiency did not prevent release of PTH or its action on the kidneys.

In 1969 Avioli et al. (0263) studied the movements of labeled D_3 in eight young adults with chronic renal failure and secondary bone demineralisation.

Absorption was normal, but plasma 25-OH-D_3 was largely replaced by inactive metabolites, and the urine contained large amounts of both D_3 and 25-OH-D_3. Long-term intermittent dialyses had no effect, but the patients who had kidney homotransplants became normal.

Similar results were obtained in experiments on rats.

The authors "tentatively" concluded that the underlying defect was either in conversion of D_3 to 25-OH-D_3 with accelerated degradation or both, or at the site of CaBP synthesis.

In 1970 Arnaud et al. (0229) reported three cases of "vitamin D dependency" in a large, inbred, French-Canadian family. They inferred an autosomal recessive transmission but admitted that the phenotypes of the presumed heterozygotes failed to support this.

Each patient had rickets with low blood P, Ca, Cl, and high blood amino acid levels. Each responded only to very high D doses, respectively 25,000 IU D_2, 45,000 IU D_2, and 45,000 IU D_3. Serum PTH was high at start but became normal during treatment or after a Ca infusion.

In 1970 Scriver (5200) listed additional manifestations of "vitamin D dependency" as muscular weakness, convulsions, and kidney tubular acidosis. He explained that "dependency" meant that patients would probably need high intakes of D for their lifetimes.

He inferred that such patients could make CaBP, and speculated that the genetic error was either in the nuclear membrane binding site for 25-OH-D, or in the mechanism for elaboration of 25-OH-D.

In 1970 Hamilton et al. (2365) demonstrated impaired Ca absorption in the intestine of a child who had normal serum D activity and D-dependent rickets. Phosphate balance was near-normal. After healing the rickets with massive D therapy, Ca absorption became normal. In five other cases microscopy of the mucosal cells revealed no morphological abnormality, and the authors inferred a defect either in metabolism of the vitamin or in elaboration of the protein carrier for Ca.

In 1970 Rasmussen and Pechet (4759) reviewed the history of the discovery and elucidation of calcitonin. Their viewpoint was that since D assisted Ca absorption from the gut, and since PTH assisted Ca mobilisation from bone and reabsorption at the kidney tubules, the serum would become supersaturated; therefore a further hormone was postulated to protect against persistent hypercalcemia.

This review is just outside the scope of the monograph but has been added for two reasons. Detailed knowledge of calcitonin is recent and may not be familiar to some readers, yet it is taken for granted in many papers that are properly included here. Also, specifically, the review explains the relevance of the hydroxyproline clearance tests (see 4235, 4236). The review is not further summarized in the monograph but may be read in full in the original.

In 1973 Fraser et al. (1898) studied five infants with D-dependent rickets, described as a recessively inherited form of D-refractory rickets. Despite normal D intakes, these patients all had early onset of low serum Ca and P, excessive serum APase, excessive urinary amino acids, and very severe rachitic bone lesions.

The bone lesions responded only to massive doses of D_2 (1.25-2.5 mg/day, or 50,000-100,000 IU), or D_3 (1.25 mg/day, 50,000 IU), or 25-OH-D_3 (0.4-0.9 mg/day, 16,000-36,000 IU). However, the lesions responded to 1 µg (40 IU) of 1α,25-$(OH)_2$-D_3 per day, which the authors believed to be a physiological amount.

From these results they concluded:

1. The target cells for D activity were responsive.

2. Conversion of D_3 to 25-OH-D_3 was normal.

3. Conversion of 25-OH-D_3 to 1α,25-$(OH)_2$-D_3 was inhibited so that massive amounts of precursor were required for elaboration of minute amounts of endproduct.

4. Therefore, the genetic defect was in the induction or structure of the enzyme 25-OH-cholecalciferol-1-hydroxylase, and the condition was truly an inborn error of metabolism of D_3.

H. Effects on the Cardiovascular System

In 1965 Zemplenyi and Mrhova (6354) fed rats so as to induce multiple thromboses and myocardial infarctions. In enzyme studies of the early phase of induction they found decreases of TCA cycle enzymes, and also decreases of acid and alkaline phosphatases and 5'-nucleotidase.

They then repeated this experiment, adding 30,000 IU of D_3 in oil daily for 5 or 9 days (6353). They found additional connective tissue damage, followed by increases of both phosphatases and 5'-nucleotidase. These increases were evident on the 9th day of the experiment but not on the 4th day.

The authors inferred that these enzyme activity increases were related specifically to the connective tissue damage, though the nature of the relationship was uncertain.

In 1970 Harrand and Hartles (2414) reported on different levels of Ca and P in the diet with and without D_2 when the Ca:P ratio was 1:1. In addition they summarized earlier studies (cited) in which the Ca:P ratio had been 1:10 and 10:1. Their studies were on the formation of bones and teeth in rats, and the original papers should be consulted for details.

Their main conclusions were:

1. Lack of minerals at all three ratios affected bone more than teeth.
2. Least effect on teeth was at Ca:P 10:1. Least effect on bone was at Ca:P 1:10.
3. Tooth mass was diminished more by lack of Ca than of P; the converse was observed for bone mass.
4. When Ca was normal (0.32% in diet) a 1:10 Ca:P deposited less mineral in bones and teeth than when Ca was 0.33%. D_2 protected bone and partly protected teeth so that excess P was worse for teeth than for bone. Excess Ca at a 10:1 Ca:P ratio had no such effect.
5. The D_2 effect was highly significant when the Ca:P ratio was 1:10, less significant when it was 1:1, and not significant when it was 10:1.
6. Nevertheless at the 10 1 ratio D_2 markedly improved the quality of both bones and teeth, and the authors suggested that D_2 improved the organization of calcifying osseous tissue in some manner that was independent of its quantitative effects on mineral deposition.

I. Effects on Metals

1. Magnesium

In 1955 Maintzer and Steenbock (3877) reported experiments on 90 g rats showing that Mg carbonate, phosphate, or phytate were equally absorbed when fed as 0.12% of a semisynthetic diet low in P and Ca. The range of absorption in the absence of D was slightly but constantly higher when D was fed than when it was absent. There was no effect on the absorption of P.

In 1961 Hanna (2382) gave two groups of female hooded rats (150-170 g) a diet with 288 μEq Mg and 2400 μEq Ca daily. One group received i.m. 40,000 IU D_2 daily for 3 days. Urinary Mg increased, fecal Mg decreased correspondingly,

and plasma Mg also decreased. The author concluded (from discussion) that Ca and Mg absorption were citrate-dependent.

In 1965 Richardson and Walt (4818) fed two groups of rats a Mg-deficient diet, and gave one group injections of D_2. This lowered serum Mg levels without affecting carcass, muscle, urinary or fecal Mg. The authors inferred that D_2 either diminished binding of Mg by serum proteins or redistributed Mg within the body, perhaps promoting its deposition in bone.

In 1966 Leeson and Fourman (3449) reported two cases in which severe parathyroid deficiency with tetany was treated with massive doses of D_2 and D_3; both patients were "accidentally (sic) poisoned," and afterwards both responded to one-tenth the doses of D that previously had failed to elicit responses.

In one case the hypercalcemia led to acute pancreatitis, in the other to renal failure; in both, the poisoning was attributed to accumulation of D in the body.

In one case the hypercalcemia was associated with low serum Mg and epilepsy; in the other, parathyroid extract lowered the urinary excretion of Mg. Both of these effects were considered secondary to the overdoses of D.

In 1967 Lifshitz et al. (3521) studied the effects of single oral doses of 5000 IU D_2 on the serum, bone, and urinary Mg of D-deficient rats fed either Mg-deficient or control diets. The doses diminished the serum and bone Mg and increased the urinary Mg of both groups, but more so the Mg-deficient group. The authors stated that this effect was opposite to that of parathyroid hormone and concluded that either it was secondary to the effect of D on the parathyroid or it was a separate effect of D on the kidney tubules.

In a further report in the same year the same authors (3520) measured Ca transport, and serum Ca and citrate, in rat gut in vitro, from Mg-deficient and control rats that were also D-deficient or D-fed. They found that Mg-deficient rats were less responsive to physiologic doses of vitamin D than were control rats.

In 1972 Seelig (5219) concluded that large intakes of D could cause excessive urinary losses of Mg and thereby depress the activities of Mg-dependent enzymes in kidneys, cardiovascular and other systems.

2. Zinc

In 1966 Becker and Hoekstra (0429) found that oral-Zn^{65} retention by rats was increased by vitamin D_2 when diets were Zn-supplemented but not when diets

were Zn-deficient. Since injected Zn^{65} was uninfluenced, the authors inferred that D_2 had improved Zn absorption. Then they found that the D_2 effect was most pronounced in D-depleted rats; it occurred in bone but not in soft tissue, and again only with oral Zn^{65}. They concluded that this effect was secondary to the effect of D on skeletal calcification.

In 1967 Leaver (3419) found in weanling rats that uptake and release of zinc by bones, normally rapid, were retarded by Ca depletion and restored by vitamin D (type not stated).

3. Copper

In 1964 Gude et al. (2283) found, in 15-20 day experiments on young rats, that large doses of vitamin D resulted in shifts of Cu distribution through the organs: +250% in bone, +60% in spleen, +30% in heart and brain, and reduced content in liver and gut. They concluded that such a redistribution was "unfavorable" and, compared with effects of vitamn A and B_1, specific to each vitamin.

4. Manganese

In 1947 Couch et al. (1168) compared the dietary requirements of pullets and hens, for egg-laying, in terms of Mn and vitamin D (form not stated). They found that pullets required only 41 ppm Mn, while hens required 71 ppm. Pullets required 38-76 AOAC chick units of vitamin D, hens required 76 or more units. When excess Mn was fed, its levels in the diet and in dried egg yolk were related, and D level variations did not affect eggshell quality, but deficiency levels of D in the diet affected the utilization of Mn.

J. Effects on Vitamins

In 1954 Raiha and Forsander (4691) measured blood cocarboxylase activity in children as an indicator of capacity to phosphorylate thiamine to its active form in the body.

They found that injections of vitamin D increased this capacity in children, and that oral or parenteral vitamin D was similarly effective in rats.

In 1959 Nose (4319) reported that in rats, induced rickets was accompanied by decreased thiamine levels in liver and blood. Injections, i.m., of thiamine and D restored thiamine levels, but thiamine alone did not. The author commented that D probably caused the thiamine retention.

K. Estrogen-like Actions of D

In 1934 Dodds (1522) reported the production of estrus in spayed rats by a variety of substances with condensed carbon ring systems, and especially by

substances with the phenanthrene nucleus, including several carcinogenic hydrocarbons. He then found that 20% of the rats injected with 100 mg of ergosterol or calciferol also developed estrus, but cholesterol was ineffective. He acknowledged that these doses were far above the antirachitic doses, and suggested that the estrogenic potencies of such compounds increased with their degrees of unsaturation.

In 1961, Oniwa (4382) carried out experiments to study the relationship of D to sexual function. The following experiments were carried out:

1. Castration and vitamin D deficiency:
    a. A D-deficient diet was given to young castrated white rats for 90 days and the same diet plus a daily injection of 50 IU of D was given to controls.
    b. The same diet was given to mature castrated white rats for 90 days with the controls receiving the diet plus 50 IU D daily.

It was observed that both young and mature D-deficient animals showed pronounced uterine atrophy. Some of the young, castrated controls had an enlarged uterus and complete cornification of the vaginal smear.

2. Large and small D doses to non-castrated young rats:
    a. Four groups of white rats (40 days old) were respectively injected with 1,000, 2,000, 10,000 IU, and sesame oil only, at one-day intervals
    b. Two groups of young white rats (45 g) were respectively injected with 200 IU D and sesame oil alone for 25 days.

The results of administering large D doses were:

    a. At the 1,000 IU and 2,000 IU doses, there was no difference in the time of vaginal dilatation as compared to the controls.
    b. No sexual cycle occurred in the rats receiving 10,000 IU of D. Both the ovary and the uterus were atrophied.

Vitamin D at the lowest level, 200 IU, stimulated uterine growth and slightly enlarged the uterine cavity.

3. Small and large vitamin D doses to castrated young rats:

Three groups of young castrated rats (45 g) were respectively administered 200 and 1,000 IU D and purified sesame oil to controls.

The 200 IU group showed marked uterine hypertrophy whereas the 1,000 IU group showed pronounced uterine atrophy. This latter group indicated an early shift to prolonged diestrus with incomplete cornification seen in the vaginal smear. At the lower dose (200 IU) no difference in vaginal dilatation time was noted between noncastrated and castrated groups.

The author concluded:

1. In castrated white rats D deficiency caused pronounced uterine atrophy. Vitamin D administration prevented this and in non-castrated rats enlarged the uterus.
2. A moderate D dose stimulated ovarian follicle growth and uterine growth. An excessive dosage along with prolonging the duration of treatment, produced a negative effect inhibiting sexual function.
3. A large D dose stimulated the sexual activity of young animals, but prolonged treatment even following vaginal dilatation inhibited sexual function before it affected their general condition.
4. Young rats were more affected by D than mature rats.

In a 1968 review Norman (4307) pointed out that a physiological dose of D was only 5-10 IU, but he was referring to rats. He described the action of D as "hormone like", and emphasized its effects on transcription of RNA from the genome.

In 1973 Jensen and DeSombre (2939) reported a mechanism for interactions between estrogens and uterine cells. They found that an estrogen became complexed with a receptor protein, and that both then migrated to the cell nucleus, depending on temperature. The receptor's sedimentation rate was increased from 3.8S to 5.2S, and it became able to bind to a cell nucleus and to augment RNA synthesis. These properties of the receptor were acquired only on its association with the estrogen.

In 1974 O'Malley and Means (4339) presented further detailed evidence confirming and amplifying the above (2939). They emphasized the variety of responses of target tissues to steroids, and that all of them involved transcription, after the steroid-protein complex had migrated to the cell nucleus. Then, according to the authors, the hormone-induced RNA (usually mRNA) returned to the cytoplasm and translation followed. Lastly came the "functional response" typical of the steroid and target tissue.

The authors concluded, from their data on estrogens and from the literature, that a primary effect of all steroid hormones was "a specific regulatory effect on nuclear RNA metabolism." Although this paper did not mention the vitamins D, its conclusions were generalized to the class of hormones to which the hormonal forms of the vitamins D have been shown to belong.

In 1974 Kenny et al. (3102) reported that ovulation in laying hens enhanced the production of $1,25\text{-}(OH)_2\text{-}D_3$ from $25\text{-}OH\text{-}D_3$.

V. Drug Interactions

A. Metabolic Inhibitors

In Vitro

In 1962, Sallis and Holdsworth (5005) investigated the effect of D on calcium absorption in the chick. White Leghorn cockerels newly hatched were fed a rachitogenic diet for four to five weeks. Some of them were then given a diet supplemented with D_3. Positive controls were administered 100 IU of D in arachis oil before the tests. To identify the source of the energy used in Ca transport, subgroups were given various metabolic inhibitors, as shown in Table 77. The results are expressed as percent of inhibition of the active transport process, i.e., the difference between D_3-treated and rachitic Ca transport. The greatest effect was obtained with a glycolysis inhibitor, 2×10^{-3}M iodoacetate, under anaerobic conditions.

Table 77

Effect of Inhibitors on Active Transport of Ca (5005)

Inhibitor and Conc., M	No. of Birds	% Inhibition
H_2/CO_2 in gas space	7	37
Sodium cyanide, 5×10^{-4}	4	8
Sodium cyanide, 1×10^{-2}	4	39.6
2\4 Dinitrophenol, 2×10^{-4}	8	52
Sodium iodoacetate, 2×10^{-3}	8	80
Sodium fluoride, 2×10^{-3}	7	54
Sodium arsenite, 2×10^{-3}	4	55
Mercuric chloride,* 2×10^{-3}	4	95
Ouabain, 6.8×10^{-5}	8	0

Active transport was taken to be the difference between Ca transported into serosal fluid by everted distal sacs from rachitic chicks as compared with similar sacs from chicks treated with 100 IU 16 hr previously. Inhibition is expressed as a percent of this active transport. The inhibitors were added to mucosal fluid and are recorded as final concentrations.

* Cells sloughed off.

B. Vitamin A

1. Mice

In 1933, Robertson et al. (4854) compared the growth rate and longevity of white mice fed a moderate overdosage of D or of A and D combined. Three groups each of 36 animals were fed a mixed diet. Group M was given a daily supplement of 50 rat-units of D dissolved in 0.05 ml olive oil; Group O received 50 rat-units of D plus 460 rat-units of A dissolved in 0.05 ml olive oil daily; Group M, which acted as normal controls, received only 0.05 ml olive oil daily.

Table 78 shows that life expectancy was greatest in the control group, less in the D-supplemented group, and least in the group given lifetime overdoses of both A and D. The authors concluded that the combination of vitamin overdoses had diminished life expectancy significantly.

2. Rats

In 1961, Clark and Bassett (1056) reported their investigations of the combined effects of A and D on rats. Male albino rats (Holtzman strain) were given A palmitate and calciferol in sesame oil daily by stomach tube. The experimental plan was:

Experiment 1 for five weeks: Five groups of eight rats each (120 to 150 g) as follows: Group I, controls; Group II, 60,000 units D; Group III, 30,000 units A; Group IV, 60,000 units D plus 15,000 units A; Group V, 60,000 units D plus 30,000 units A.

Experiment 2 for 15 days: Four groups of 15 rats each (100 to 150 g) as follows: Group I, controls; Group II, 60,000 units D; Group III, 30,000 units A; Group IV, 60,000 units D plus 30,000 units A.

Experiment 3 for 60 days: Five groups of six rats each (85 to 100 g) as follows: Group I, controls; Group II, 18,000 units D; Group III, 18,000 units D plus 300 units A; Group IV, 18,000 units D plus 3,000 units A; Group V, 18,000 units D plus 30,000 units A.

In the first two experiments (Tables 79 and 80) the control groups had the best growth and survival records, but when A was added to D:

a. Survival time was increased.

b. Weight loss was unaffected.

c. Less skeletal damage was observed.

d. When 30,000 units of A were given with D, little or no kidney calcification was seen.

e. There was no significant myocardial damage.

Table 78

Mortality Statistics (4854)
(Accidental deaths excluded)

At Age in Days	Percentage of Survivors Group M Control	Group N Vitamin D	Group O Vitamins A + D
200	100	100	100
250	100	100	97.2
300	100	100	97.2
350	100	100	97.2
400	100	97.1	91.7
450	100	97.1	83.3
500	100	94.3	80.6
550	100	85.7	80.6
600	94.3	85.7	75.0
650	82.9	82.9	69.4
700	71.4	74.3	63.9
750	62.9	62.9	58.3
800	60.0	42.9	58.3
850	48.6	37.1	52.8
900	34.3	37.1	41.7
950	20.0	28.6	22.2
1,000	11.4	14.3	16.7
1,050	5.7	11.4	8.3
1,100	5.7	5.7	0.0
1,150	2.9	0.0	
1,200	0.0		

Mean Duration of Life

Group M 825 ± 17 days
Group N 806 ± 22 days
Group O 771 ± 26 days
Difference between M and O 54 ± 31 days

Mean Duration of Life of Mice Still Alive at 750 Days
Group M 924 ± 14 days
Group N 920 ± 17 days
Group O 944 ± 11 days
Difference between M and O 20 ± 18 days

Mean Duration of Life of Mice Dying Before 750 Days
Group M 658 ± 9 days
Group N 612 ± 21 days
Group O 529 ± 24 days
Difference between M and N 46 ± 23 days
N and O 83 ± 32 days
M and O 129 ± 25 days

Table 79

Effect of 60,000 Units of Vitamin D with and without Vitamin A on Body Weight and Rat Mortality (1056)

Treatment	No. of Animals	Weeks on treatment						No. dead at 5 weeks
		0	1	2	3	4	5	
		gm	gm	gm	gm	gm	gm	
Control	8	137 (0)*	177 (0)	217 (0)	254 (0)	275 (0)	303† (0)	0
60,000 D	8	138 (0)	107 (0)	98 (0)	90 (0)	90†		7
30,000 A	8	137 (0)	166 (0)	194 (0)	219 (0)	252 (1)	286† (0)	1
60,000 D + 15,000 A	8	138 (0)	118 (0)	100 (0)	90 (0)	90† (4)		7
60,000 D + 30,000 A	8	138 (0)	121 (0)	101 (0)	91 (0)	83 (1)	81* (3)	4

* The numbers in parentheses refer to the number of rats which died between weighing periods; the other numbers refer to the average body weight.

† Sacrificed for histology.

Table 80

Effect of 60,000 Units of Vitamin D with and without 30,000 Units of Vitamin A on Body Weight and Rat Mortality (1056)

Treatment	No. of Animals	Days on treatment									
		0	4	7		11		15			
		gm	gm	gm		gm		gm			
Controls	15	133	142	158		179		191			
60,000 D	15	129	111	109	(3)*	98	(2)*	96	(3)*		
30,000 A	15	131	139	152		162		171			
60,000 D + 30,000 A	15	133	110	110		101		101			

* The numbers in parentheses refer to the number of rats which died between weighing periods; the other numbers refer to the average body weight.

The growth curves for experiment 3 are shown in Figure 13. The addition of 30,000 units of A prevented significant weight loss, pathologic changes in skulls and tibiae and severe kidney calcification. The smallest supplementation, 300 units of A, had little or no positive effect while 3,000 units of A had an intermediate effect. The authors concluded that administration of relatively large amounts of A to rats with hypervitaminosis D decreased the toxicity of D.

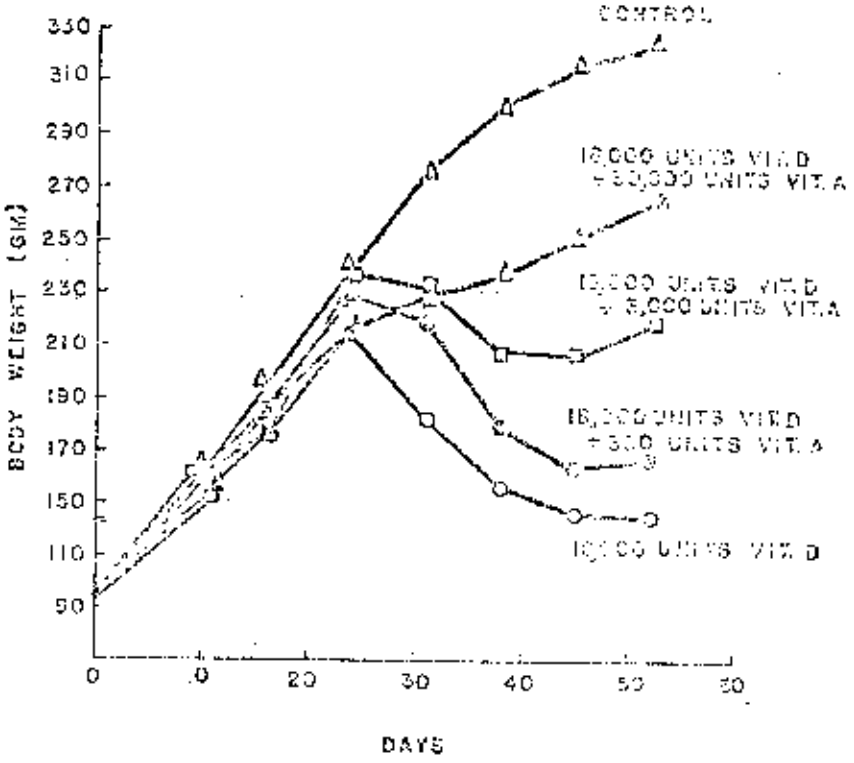


Figure 13. Growth curves of rats which survived the 6 week period. (1056)

3. Chicks

In 1968, Taylor et al. (5718) investigated the possibility of an antagonism caused by excesses of A and D in chicks. Groups of twelve male chicks (Thornber '404' strain) were fed experimental diets containing various levels of the two vitamins. Weight changes and plasma levels of Ca and P_i are shown in Tables 81, 82, and 83 for the first experiment.

A second experiment in which these vitamins were given at 1000 times the basal levels showed that:

Table 81

Expt. I: mean live weights (g) of chicks at 5 weeks of age given four levels of vitamin A and four levels of vitamin D (5718)

(Means for ten chicks/treatment)

Level of vitamin A (X basal level)	Wt with level of vitamin D (X basal level) of: 1	10	100	1000	Mean wt.
1	438	459	445	264	402
10	482	474	425	289	418
100	449	493	456	368	442
1000	309	239	276	278	275
Mean	419	416	401	300	---

SE of the difference between means in the body of the table = 24'2.
SE of the difference between vitamin A and vitamin D means = 12'1.

Table 82

Expt. I: mean values for concentration (mg/100) of plasma calcium for Chicks, 4, 5, and 6 weeks of age given four levels of vitamin A and four of vitamin D

(Means for six chicks/treatment) (5718)

Level of vitamin A (X basal level)	Concentration with level of vitamin D (X basal level) of: 1	10	100	1000	Mean Concentration
1	11'0	10'5	11'3	13'1	11'5
10	10'8	10'8	10'9	13'1	11'4
100	10'6	11'2	10'8	12'9	11'4
1000	9'6	10'0	10'3	10'7	10'2
Mean	10'5	10'7	10'8	12'5	---

SE of the difference between means in the body of the table = 0'57.
SE of the difference between vitamin A and vitamin D means = 0'29.

a. Weight gain was depressed whenever either or both vitamins were fed at 1,000 times the basal level.

b. Increasing amounts of A administered with the highest level of D caused a progressive increase in plasma P_i.

c. An excess of D, added to a basal amount of A, depressed the activity of plasma acid phosphatase ($P<0.001$), and this was counteracted progressively by increasing the amount of A (Table 84).

C. Strontium

In 1973 Omdahl and DeLuca (4376) reported that chicks fed a strontium diet developed rickets with deficiencies of Ca absorption and CaBP synthesis. They metabolised doses of D_3 to 24,25-$(OH)_2$-D_3 instead of to 1,25-$(OH)_2$-D_3, and they responded, in terms of improved Ca absorption, to doses of 1,25-$(OH)_2$-D_3 and not to 25-OH-D_3. The response in terms of bone mineralization remained to be studied.

In 1974 Wasserman (6122) reported that this effect of strontium in chicks was counteracted by a water-soluble factor isolated from the plant, *Solanum malacoxylon*, found in Argentina. The factor, not yet identified, had an activity similar to that of 1,25-$(OH)_2$-D_3 and had caused soft-tissue hypercalcification in cattle at pasture. The author concluded from his experiments in chicks that strontium inhibited 1-hydroxylation of 25-OH-D_3 in the kidney.

D. Lactose

In 1965 Dupuis and Fournier (1595) investigated a possible antirachitic relationship between D and lactose. Laboratory-bred Wistar rats, 13 groups of six, were dissected after three weeks on various diets:

1. A diet with a Ca utilization factor.
2. The same, supplemented with calciferol in varying amounts.
3. As diet 1, but with lactose in varying amounts replacing starch.

The results are summarised in Table 85, and the authors concluded that:

a. In the young rat, lactose and D possessed similar antirachitic properties.

b. At small dosages, the antirachitic effects of lactose and D were additive.

Table 83

Expt I: mean values for concentration (mg/100 ml) of plasma inorganic phosphorus for chicks 4, 5, and 6 weeks of age given four levels of vitamin A and four of vitamin D (5718)

(Means for six chicks/treatment)

Level of vitamin A (X basal level)	Concentration with level of vitamin D (X basal level) of: 1	10	100	1000	Mean concentration
1	7·6	7·5	8·0	3·8	6·7
10	7·6	8·2	7·9	4·6	7·1
100	8·2	8·4	8·5	5·4	7·6
1000	7·4	7·8	8·0	8·1	7·8
Mean	7·7	8·0	8·2	5·4	---

SE of the difference between means in the body of the table = 0·62.
SE of the difference between vitamin A and vitamin D means = 0·31.

Table 84

Mean activities (i.u.) of plasma acid phosphatase in chicks 5 and 6 weeks of age in Expt I and 4 and 5 weeks of age in Expt 2 (5718)

(Means for nine or ten chicks/treatment)

Level of vitamin A (X basal level)	Activity with level of vitamin D (X basal level) of: 1	10	100	1000
1	28·1	--	--	16·9***
10	--	--	--	21·0‡
100	--	--	--	27·8‡
1000†	47·4***	48·4***	47·1***	44·9***

*** Significantly different ($P < 0·001$) from control (1 A, 1 D) means.
† No significant differences within these four treatments.
‡ Significantly different ($P < 0·001$) from 1000 A means.

Table 85

Results of Experimental Diets fed to Rats (1595)

Diets + Lots		Calcium in blood in mg/l	Characteristics of Tibias			Degree of Rickets
Characteristics	#		Consistency	Cartilage	Ossification Zone	
No factor	1	60	soft	very thick and very irregular	areas are rare and twisted	6
Lactose 2%	6	68		thick,	areas very	
Vit. D 1/16 ...	2	68	fairly	forming one	irregularly	4
Lactose 4%	7	70	soft	or two large sacs	placed	
Vit. D 1/16 + Lactose 2%	10	76	firmer	very large + irregular in spots	poorly organised across from cartilage irregularities	3
Vitamin D 1/16 + lactose 4% ...	11	89		often a	generally well distributed	from
Vit. D 1/8	3	87	fairly	little too	except where	2 to 1
Lactose 8%	8	85	hard	big at the	cartilage is	
Vit. D 1/8 + lactose 2% ...	12	85		edge	enlarged	
Vit. D 1/8 + lactose 4% ...	13	98		essentially normal	fine and parallel zones	from
Vit. D 1/4	4	94	hard	thickness +		1 to 0
Lactose 16% ...	9	96		regularity		
Vit. D 1/2	5	101	very hard	ends are edged + nacreous	well ordered	0

E. Steroids

1. Cholesterol

In 1933 Harrison (2440) found that D and cholesterol, fed to rabbits, each produced its own lesions in the aortas. Twenty-four one-year-old rabbits were given D alone, or cholesterol alone, or D then cholesterol, or cholesterol then D, as in Table 86.

Lesions were produced in all animals. When cholesterol was given first, D produced additional lesions. When D was given first, cholesterol produced additional lesions. In both cases the additional lesions were seen in parts of the aorta untouched by the first treatment.

In 1957 Donath and de Langen (1533) fed rabbits a stock diet and gave them either 25 mg cholesterol in 2 ml oil daily for 250 days, or this plus 5 drops of D_3 (Vigantol) and about 1 mg irradiated ergosterol for the first 10 to 25 days.

According to the authors, the results confirmed a previous finding that D_3 and cholesterol were synergistic for arteriosclerosis, especially in older rabbits.

2. Cortisone

In 1957 Cruickshank and Kodicek (1239) studied the effect of cortisone acetate (CA) on rats with hypervitaminosis D. Three groups of eight rats (70 g) were fed a rachitogenic diet for five weeks, and, per rat:

Group 1: 1 mg D_2 in arachis oil daily by mouth.

Group 2: Same plus 1 mg CA daily.

Group 3: 1 mg CA daily i.m., and approximately 1.25 µg D_2 weekly (control group).

The results are summarized in Table 87 and Figure 14. Some of the observations were:

a. All of group 1 lost weight and were in poor condition, and two died.

b. The loss of weight and condition was even worse in group 2, and six died.

c. All of group 3 thrived.

d. High doses of D_2 increased urinary excretion of P, whether CA was given or not.

e. Somewhat more bone ash was found in the controls than in the other two groups.

f. The bones of the controls appeared radiologically normal, whereas the groups 1 and 2 rats showed varying degrees of circumscribed osteoporosis

Table 46

Experimental Protocol and Results (2440)

	No.	Sex	Initial weight in gms	Age in months	Treatment	Duration of cholesterol treatment	Duration of irradiated ergosterol treatment	Killed or died	Intimal aortic lesions	Medial aortic lesions	Intimal lesions in other vessels	Medial lesions in other vessels	Renal calcification
						First Experiment							
A	1	F	1800	12	Cholesterol	90 days	62 days	Killed	+	+	+	...	+
	2	F	1590	14	1 gm per day	90 days	62 days	Killed	+	+	+	+	+
	3	M	1720	13	followed by	90 days	14 days	Died	+	+	+	...	+
	4	M	1320	11	irradiated	90 days	15 days	Died	+	+	+	...	+
	5	M	1750	12	ergosterol	90 days	33 days	Died	+	+	+	+	+
	6	M	1950	12	100,000 units per day	90 days	16 days	Died	+	+	+	...	+
B Control	7	M	1930	15		83 days	...	Died	+	...	+	...	...
	8	F	2280	15		83 days	...	Died	+	...	+	...	...
	9	M	1890	13	Cholesterol	90 days	...	Killed	+	...	+	...	...
	10	M	1730	12	1 gm per day	90 days	...	Killed	+	...	+	...	...
	11	M	1600	12		90 days	...	Killed	+	...	+	...	...
	12	M	1670	13		90 days	...	Killed	+	...	+	...	...
C Control	13	F	1930	11		...	14 days	Died	...	+	...	+	+
	14	F	1530	12	Irradiated	...	62 days	Killed	...	+	...	+	+
	15	F	2190	17	ergosterol	...	16 days	Died	...	+	...	+	+
	16	F	1660	16	100,000 units	...	21 days	Died	...	+	...	+	+
	17	M	1510	11	per day	...	21 days	Died	...	+	...	+	+
	18	M	1630	11		...	18 days	Died	...	+	...	+	+
						Second Experiment							
	19	M	1200	10	Irradiated	83 days	11 days	Killed	+	+	+	+	+
	20	M	1550	12	ergosterol	83 days	11 days	Died	...	+	...	+	+
	21	F	2140	14	100,000 units	83 days	11 days	Killed	+	+	+	+	...
	22	F	1640	12	followed by	83 days	11 days	Killed	+	+	+	...	+
	23	F	1940	12	cholesterol	83 days	11 days	Killed	+	+	+	+	+
	24	M	1900	13	1 gm per day	83 days	11 days	Killed	...	...	+	...	+

Table 87

Effect of Cortisone Acetate (CA) on Toxicity of Vitamin D_2 in Rats (1239)

Daily dose (mg)	No. of rats	Deaths	Avg. weight gain or loss (g)*	Urinary P** (mg/rat/day)	Bone ash (%)	Calcification in organs
I. Vitamin D_2, 1	8	2	- 6	3.0	41	+ +
II. Vitamin D_2, 1+CA, 1	8	6	-18	2.7	43	+ +
III. CA, 1	8	0	+13	0.14	48	0

* Change in weight during experimental period of 5 weeks.
** Determined at end of experiment.

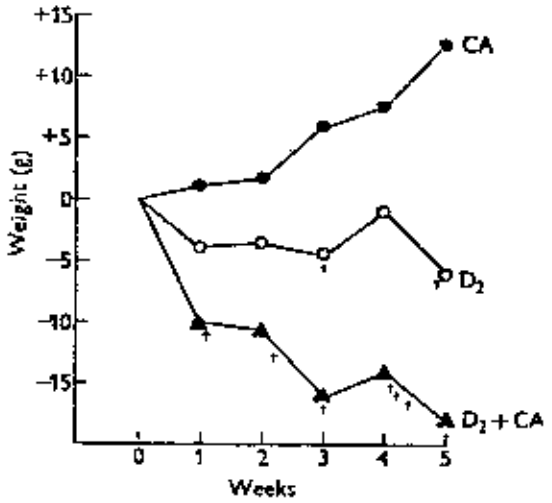


Figure 14. Effect of cortisone acetate (CA) on average growth of hypervitaminotic (D_2) rats. (1239)

† = Death of animal.

indicating a disturbance of mineralization. No gross calcification of soft tissue was seen.

g. On microscopy, deposits of Ca salts, in the endothelial layers of the aorta and in the kidneys, were found in groups 1 and 2 but not in the controls.

The authors concluded that in rats with hypervitaminosis D, cortisone did not counteract the weight loss, clinical appearance, histological lesions or increased urinary P excretion. This finding was in contrast to man, in whom the beneficial effect of cortisone on the syndrome of D toxicity had been demonstrated.

In 1973 Omdahl and DeLuca (4376) commented in a review that the action of corticosteroids on D intoxication was not yet clear. In man, these steroids decreased the hypercalcemia of infancy, in sarcoidosis, or from overdosage of D. The absorption of Ca was diminished, but the effects of the metabolism of D were equivocal. In rats, the level of D-dependent CaBP was increased. The discrepancy between diminished Ca absorption and increased CaBP "has not been resolved" (4376).

3. Estrogen

In 1961, Oniwa (4382) studied the interactions of D and estrogen in female rats. Four experiments were performed as follows:

(1) Four groups of mature castrated white rats were administered respectively 20 IU of ovarian hormone (OvH) twice daily for five days alone; 1000 IU D in addition to the OvH once daily; 5000 IU similarly; 10,000 IU similarly.

No significant difference was noted in the duration of estrus among the four test groups.

(2) Two groups of mature castrated rats were administered respectively 1000 IU D daily for 20 days followed by 20 IU OvH twice daily for five days; sesame oil for 20 days followed similar OvH administration as in the first group.

No appreciable difference in the duration of estrus between the two groups was noted.

(3) Two groups of noncastrated young white rats (45 g) were administered respectively daily for 20 days 100 IU of OvH only; 100 IU of OvH plus 200 IU of D.

The second group showed superior uterine growth and more corpora lutea in the ovaries.

(4) Three groups of castrated young white rats (48 g) were administered respectively daily for 25 days 100 IU of OvH; 10,000 IU of OvH plus 200 IU of D; 100 IU of OvH plus 200 IU of D.

The uterus was enlarged in all groups but least in the group given OvH alone. Cornification occurred slightly earlier in the two groups given OvH plus D.

The author commented that small amounts of D intensified the action of estrogen, whereas large amounts were, if anything, inhibitory under these conditions.

F. Parathyroid Hormone (PTH)

In 1964 Toverud (5827) investigated the effect of D on the Ca mobilizing action of PTH in rats bred from hooded and albino strains. A D-deficient diet was fed, and some rats were parathyroidectomised. PTH containing 375 units/ml was given s.c., and D_3, 2000 IU, was given orally in arachis oil.

From the data in Tables 88, 89, 90, 91, 92, and 93 the author concluded that:

1. In this strain of rats, the Ca-mobilising action of PTH was either independent of D or dependent only on minute amounts, which might be present in severely rachitic rats.
2. When intestinal Ca absorption was minimal, no evidence for interaction between the effects of D and PTH was found at the dose level investigated.

In 1973 Omdahl and DeLuca (4376) commented that evidence on the degree of interdependence of D and PTH was conflicting, and more work was needed on the relationships of these and calcitonin in renal function. D had been shown to increase tubular reabsorption of Ca and P, while PTH had been shown to induce a P diuresis. D had also been shown to induce P diuresis, but only in large doses. Workers disagreed on whether the P diuresis from PTH was D-dependent. The authors suggested that experiments with the new D metabolites and analogs ought to resolve these conflicts in due course.

G. Diphosphonates

In 1970 Fleisch et al. (1825) reported that phosphonates (compounds with C-P bonds), especially those with P-C-P bonds such as methyl diphosphonate, ethane-1-hydroxy-1:1-diphosphonate (EHDP) and dichloromethylene diphosphonate, acted similarly to condensed phosphates (with P-O-P bonds) in that:

(1) They could inhibit crystallisation of $CaPO_4$ in vitro, and

(2) When given orally or parenterally to rats they inhibited calcification of aorta and kidneys induced by hypervitaminosis D_3.

Tabl

Effect of vitamin D and/or parathyroid extract on the serum calcium of parathyroidectomized, vitamin D-deficient, adult female rats (5827)

Mean ± standard error of 7 rats in each group. The 24-month-old rats had been fed for 18 months the vitamin D-free stock diet with weekly supplements of carrots or spinach. The doses of vitamin D (D) and parathyroid extract (PTE) were 250 IU and 270 units, respectively. Range of body weight: 185-256 g.

Days after PTX[2]	Serum calcium (mg/100 ml)					
	Group I		Group II		Group III	
	Level	Rise	Level	Rise	Level	Rise
6	4.46 ± 0.23	—	5.20 ± 0.26	—	4.62 ± 0.18	—
10	—	—	D	D	D	D
12	PTE	PTE	—	—	PTE	PTE
13	6.67	2.20 ± 0.23	7.16	1.96 ± 0.31	8.88	4.25 ± 0.24
20	5.52	—	6.33	—	6.41	—
20	PTE	PTE	PTE	PTE	—	—
21	6.80	1.28 ± 0.17	8.15	1.82 ± 0.19	—	—
25	5.15	—	[4]6.52	—	—	—

[1] Analyzed in single 0.5 ml samples of serum according to the method of Hunson et al. (1955) without the noted modifications.

[2] Parathyroidectomy.

[3] Individual rise above the previous level.

[4] 6 rats.

Table 89

Plan of the experiment on the effect of parathroid extract (PTE) and/or vitamin D on the serum calcium of vitamin D-deficient young rats (5827)

On the day of the first bleeding six-week-old rats, having been fed the "high-protein" diet for 20 days, were given a solution of 10 per cent glucose and 0.9 per cent NaCl in de-ionized water instead of solid food and tap water.

Time before second bleeding	Group I Control	Group II PTE	Group III Vit. D	Group IV Vit. D + PTE
4 days	First bleeding	First bleeding	First bleeding	First bleeding
3 days	—	—	Vit. D[1]	Vit. D[1]
24 hrs	0.9% NaCl[2]	PTE 200 u.	—	PTE 200 u.
6-8 hrs	0.9% NaCl[3]	PTE 100 u.	—	PTE 100 u.
0 hrs	Second bleeding	Second bleeding	Second bleeding	Second bleeding

[1] 100 IU

[2] 0.50 ml

[3] 0.25 ml

Table 90

Effect of parathyroid extract (PTE) and/or vitamin D on the serum calcium of vitamin D-deficient young rats (experiment 37) (5827)

The treatment plan is given in Table 89. The mean and standard error (S.E.) of serum calcium is given as mg/100 ml.

		Group I Control	Group II PTE	Group III Vit. D	Group IV Vit. D + PTE
First bleeding	Mean	7.42	6.89	7.41	6.83
	S. E.	±0.18	±0.26	±0.26	±0.21
	No.[1]	16	11	15	13
Second bleeding	Mean	6.85	7.87	8.45	9.52
	S. E.	±0.20	±0.21	±0.12	±0.17
	No.	16	15	15	16
Individual increase	Mean	-0.57	+0.86	+1.04	+2.58
	S. E.	±0.13	±0.25	±0.17	±0.15
	No.	16	11	15	13

[1] Number of observations.

Table 91

Design of Test for Additivity of Main Effects of Parathyroid Extract (PTE) and Vitamin D (D) (5827)

	No PTE	PTE
No D	Group I $\bar{x}$ = -0.57	Group II $\bar{x}$ = +0.86
D	Group III $\bar{x}$ = +1.04	Group IV $\bar{x}$ = +2.58

$\bar{x}$ refers to the mean individual increase in serum calcium (Table 90).

Table 92

Effect of parathyroid extract (PTE) on the serum calcium of vitamin D-deficient rats consuming different diets (5827)

Mean ± standard error. Age and weight were recorded 1, 2 or 3 days before the first injection. The rats received the experimental diets ("high-protein" in experiment 38 and "low-protein" in experiment 41) from time of weaning and throughout the period of observation. The experimental rats were given injections of 200 and 100 units parathyroid extract approximately 24 and 6 hours, respectively, before bleeding. The control rats received comparable injections of physiological saline solution. Rats in experiment 41 were also bled 1 day before the first injection was given.

Exp. and group	Age (days)	Weight (g)	No. rats	Serum calcium (mg/100 ml)		
				Before treatment	After treatment	Difference
38 PTE	45	102 ± 4	17	—	8.77 ± 0.27	—
38 Control	45	101 ± 3	18	—	7.39 ± 0.29	—
41 PTE	47	81 ± 2	14	5.65 ± 0.19	7.35 ± 0.20	1.70 ± 0.15
41 Control	47	78 ± 2	13	5.80 ± 0.17	6.22 ± 0.16	0.42 ± 0.10

Table 93

Effect of parathyroidectomy (PTX) and sham operation (Sham) on the serum calcium of vitamin D-deficient young rats (5827)

Mean ± standard error. Age and weight were recorded 1, 2 or 3 days before time of the operation. The operated rats represent that fraction of a larger group (35 rats in both cases) which had the lowest serum calcium levels. The "high-protein" diet was fed to all rats throughout the experiment. The calcium content of the diet in experiment 38 was reduced to 0.52% 20 days before the operation.

Exp. and group	Age (days)	Weight (g)	No. rats	Serum calcium (mg/100 ml)			
				Before operation		After operation	Individual difference
				29 days	2 days		
39 Sham	48	106±6	6	—	8.18±0.21	[1] 7.81±0.35	-0.38±0.24
39 PTX	48	115±7	6	—	8.03±0.28	[1] 5.57±0.39	-2.47±0.36
38 PTX	76	151±6	7	6.23±0.20	6.65±0.16	[2] 3.83±0.06	-2.82±0.13

[1] 5 hours after the operation.

[2] 7 hours after the operation.

The authors commented that the phosphonates were more resistant to chemical and enzymatic breakdown than were the phosphates, and suggested therapeutic trials in man.

In another paper these authors reported that $Cl_2C(PO_3HNa)_2$ and $H_2C(PO_3HNa)_2$ retarded the dissolution of apatite crystals *in vitro*, inhibited bone resorption induced by parathyroid extract in tissue culture, and reversed parathyroid-induced hypercalcemia *in vivo* in mice after oral administration (1823).

In addition, the diphosphonates (but not a monophosphonate) completely prevented aortic calcification in rats given daily oral doses of 75,000 IU D_3; the progress of acute myositis ossificans was arrested in two human patients given $CH_3C(OH)(PO_3HNa)_2$ (1886).

In 1971, Fraser *et al.* (1896) investigated the effect of EHDP on urinary stones produced in rats by D_3. The results (Table 94) showed that EHDP 0.5% w/v inhibited the formation of the calcium hydrogen phosphate stones caused by administration of D_3 10,000 IU/week.

In 1974 Talmage and Anderson (5672) studied the effect of EHDP 40 mg/kg/day in rats without thyroids and parathyroids. These rats had been injected s.c. with PTH to give a controlled hypercalcemia.

EHDP reduced the hypercalcemia, but it also created a low blood P, considered to reflect extra excretion of P by the kidneys.

The authors inferred that EHDP affected the action of PTH on the kidneys, and therefore could affect D metabolism by the kidneys.

In 1974 Baxter *et al.* (0409) studied this inhibition in chicks *in vivo* and *in vitro*, and concluded that high doses of EHDP inhibited the 1-hydroxylation of 25-OH-D_3 by the kidneys.

H. Barbiturates

In 1973 Omdahl and DeLuca (4376) alluded to a single report that D_3 metabolism was accelerated in patients on long-term barbiturate therapy. The report included supporting data from short-term studies in rats, and associated the effect with liver microsomes. However, the authors (4376) cautioned that in their opinion, the reported experimental conditions could produce artifacts, and that judgement should be suspended until the alleged metabolites were identified.

subject to much individual variation. They believed that much but not all of the damage was secondary to D-induced damage in the kidneys, but reversibility of the vascular damage depended entirely on reversal of kidney damage.

In a long discussion on the possible relevance of rat findings to atherogenesis in man, the authors concluded that in detail their findings appeared to be relevant. However, in man they pointed to ethnic "fundamental differences in calcium and phosphorus metabolism determined by diet."

2. In 1960, Chinone (1024) studied the histological changes in the ovaries of D deficient and D overdosed albino rats. (See p.222 and p.150 for related studies by Oniwa and Kudo respectively). Table 55 shows the histological changes in D deficient immature rats. The author concluded that D-deficiency resulted in atrophy of the genitals with accompanying reduction in sexual function.

In the experiment with D overdosage the weights of ovaries of rats given 1000 IU or 5000 IU of D were greater than those of controls. Uteri in the group given 10,000 IU showed a tendency to be atrophied. The estrogenic effect of D in women has been reported by Freedman (1920).

The author concluded that a small amount of D stimulated follicular growth causing uterine thickening and acceleration of sexual function. A large, long-term dose however, caused first a transient stimulus but eventually caused the genitals to atrophy and arrested sexual function completely.

3. In 1968, Ornoy et al. (4389) investigated the effect of hypervitaminosis D_2 on the mineral composition of rat fetuses, fetal bones and placentas and on the maternal serum levels of Ca and P. A total of 24 pregnant and 12 nonpregnant albino rats (180-220 g) were administered 4000, 20,000 or 40,000 IU D_2 in 1 ml olive oil solution by intragastric intubation. Twelve controls received only olive oil. The animals were divided into eight experimental groups. The experimental results are summarized in Tables 56, 57, 58 and 59.

The significant results were:

a. Animals which received 40,000 units showed a statistically significant decrease of fetal wet weight, ash weight, and Ca and P contents (see Table 57).

b. Significant alterations in the composition of fetal bone were produced by 40,000 units. The concentrations of both Mg and P were considerably higher than controls (Table 58).

c. Placental weight was reduced in the groups receiving 20,000 and 40,000 IU D_2 (Table 59).

I. Anticonvulsants

In 1973 Omdahl and DeLuca (4376) investigated the effects of 5,5-diphenylhydantoin (Dilantin) on the metabolism of labeled D_3 and 25-OH-D_3. They were led to do so by reports (cited) of rickets in children and hypocalcemia in adults who had received anticonvulsants, and that the rickets had responded to D. In short-term experiments in rats the authors found that both D_3 and 25-OH-D_3 disappeared more rapidly from the serum of Dilantin-treated rats than of controls, but found no other effects on D "metabolism per se." They commented that long-term animal studies were needed, because the reported effects in man were long-term.

In 1974 Villareale et al. (5994) studied some interactions between D_3 and Dilantin in chicks. Day-old White Leghorn cockerels were given D-free but otherwise adequate diets, plus D_3 and/or Dilantin as in Table 95.

The Dilantin produced rickets, hypocalcemia, diminished CaBP, and diminished intestinal Ca transport. The effects were dose-related, but they were also related inversely to intakes of D_3.

The authors concluded that Dilantin acted on the metabolism of D_3 or on the tissue responses to D_3, and not upon the Ca transport mechanisms. They suggested that the chick was a good animal in which to study drugs suspected of D-antagonism. They also suggested that intakes of D should be watched in patients requiring seizure therapy.

Table 95

Effect of diphenylhydantoin on body weight, serum calcium concentration, and tibia ash of chicks on two levels of vitamin D_3. (5994)

Group*	Treatment		Terminal body weight (mg)	Serum calcium (mg/100 ml)	Tibia ash (%)‡
	D_3† (I.U./day)	DPH (mg/rat)			
1	0	0	193 ± 11	6.4 ± 1.2	26 ± 1
2	3	0	280 ± 14	9.7 ± 0.1	39 ± 2
3	3	760	258 ± 10	7.9 ± 1.1	34 ± 1
4	3	1800	198 ± 9	6.2 ± 0.2	29 ± 2
5	6	0	305 ± 13	10.0 ± 0.2	41 ± 1
6	6	760	293 ± 6	9.7 ± 0.2	42 ± 1
7	6	1800	260 ± 12	8.5 ± 0.3	36 ± 1

* Six chicks per group; values represent means ± standard error of the means. † Vitamin D_3 given orally. ‡ Percentage of fat-free dry weight.

VI. Consumer Exposure Information

A. Data from Official Compendia

1. No quantitative data on exposures of the United States population to sunlight were found in any of the compendia that were consulted. No data were found on amounts of UV incident on the land surface of the United States, or on any part of the United States, at any time of year. No such data were found in sources outside the official compendia. Thus, no base-line data were found for assessment of endogenous D activity in the US population or in any segment of it.

2. In 1971 The US Tariff Commission (5878) listed the following suppliers of D_2:

Peter Hand Foundation
R.P. Scherer Corporation
Vitamins Inc.

and of D_3:

Diamond Shamrock Corporation
Dave's Laboratories Inc.
Peter Hand Foundation
Vitamins Inc., also listed alone as a supplier of provitamin D_3 (7-dehydrocholesterol)

The Commission also listed the following turnover statistics for 1969:

US totals, bulk medicinal chemicals:

Production - 200 million lbs (12% above 1968)
Sales - 145 million lbs, value $462 million (compared with 123 m lbs value $415 m in 1968; and 127 m lbs value $385 m in 1967).

US total of vitamins:

Production 17,647,000 lbs.
Sales 14,777,000 lbs, value $71,545,000 or $4.84/lb.

US totals of vitamins D:

Production: 10,000 lbs, or 177,805 billion IU.
Sales: 4,000 lbs, or 79,597 billion IU, value $655,000,000 or $8.23/billion IU.

3. In 1972 the USDA(6394) listed the following civilian consumptions of condensed and evaporated milks:

Year	Million lbs	Lbs per capita
1929	1657	13.6
1939	2327	17.8
1947	2904	20.4 (peak)
1948	2926 (peak)	20.2
1949	2919	19.8
1959	2501	14.4
1969	1565	7.9
1971	1386	6.8

Skim milks, infant diet formulas, Mellorine were not mentioned separately.

Statistics for "all cereals" were not broken down, so no data were given on consumptions of breakfast cereals, flour, farina, bread, buns, or rolls. However, the following consumptions were listed for margarine:

Year	Million lbs	Lbs per capita
1949	851	5.8
1959	1604	9.2
1969	2154	10.8
1970	2223	11.0
1971	2264	11.1

From 1967-1971 the listed price fluctuated in the range (for yellow, colored margarine) 17.2 to 30,8¢ per lb.

4. In 1972 the NAS NRC (1120) defined vitamin D_2 as a "nutrient" and "dietary supplement", the same applying to D_3. In 1965 they (0080) had described these two substances as "nutrition factors", to be found as additives in:

> Prepared breakfast cereals, vitamin D-milk, evaporated milk, skim milk, infant dietary formula, Mellorine (vegetable-fat imitation ice-cream), and margarine.
>
> 250-1000 IU of D per lb were to be found in enriched flour, enriched bromated flour, enriched self-raising flour, enriched cornmeal and grits, enriched macaroni and noodle products.
>
> Enriched farina contained 250 IU/lb, enriched bread and rolls contained 250-750 IU/lb, and evaporated milk contained 25 IU per fl. oz. of finished product.

5. In 1972 the CRC Handbook of Food Additives (0988) stated that pasteurization, sterilization, or H_2O_2 had little effect on D in fluid milk, and that dry milk could be fortified by blending with a beadlet form of D or by homogenising with D in an oil carrier before drying. "Overages are necessary", the book stated, because of analytical errors in assaying low-potency products.

On the other hand, D_2 was the "common form used in human nutrition."

6. In 1972 the FEMA GRAS Survey published by NAS NRC (1851, 4190) estimated consumer exposures to D as shown in Tables 96-98.

B. Information from Suppliers

Inquiries were made of three major suppliers of milk to the greater Washington, D.C., area, about their policy and turnover of vitamin D-milk and vitamin D-free milk. One supplier was a retail grocery chain offering both types of milk; this supplier gave us verbal information but asked not to be put on record. The second supplier was a retail grocery chain offering only vitamin D-milk, and this supplier stated (2092):

> "During 1969, when we opened our Dairy operation, we manufactured both the plain homogenized and Vitamin D milk. There was a 5¢ spread in retails between the two milks.
>
> In 1971, the retails were the same and sales dropped on the plain homogenized making it no longer economically possible to continue to process plain homogenized milk."

The third supplier was a milk producer cooperative with 206 convenience outlets in the greater Washington area, supplying milk and general groceries. This supplier answered (0903) that their policy was to offer the customer a choice where possible, and that the relevant results were as in Table 99.

In February 1973, the Upjohn Company, having acquired a licence to develop some of the new metabolites and analogs of D_3 mentioned earlier in this monograph, described the current position as follows (5895):

> "Questions have been raised concerning the current status of the active metabolites of Vitamin D_3, 25-OHD_3 and 1,25-$(OH)_2D_3$, and the closely related analog, 1$\alpha$$OHD_3$. In support of basic research by Dr. DeLuca and by clinical scientists working in the field, The Upjohn Company, Kalamazoo, Michigan, prepared a Master File, completed 6-month toxicology in the rat and dog, and provided unit dosage forms to investigators who had effective INDs, with approval of the FDA. More recently, they have filed an IND and are sponsoring clinical studies to establish conditions under which 25-OHD_3 is safe and effective in treating specific indications which have represented problems in the use of available forms of the vitamin.

Table 96. Usage Levels Reported (1851)

Substance	Food Category	Number Firms Reporting	Wtd. Mean, % Usual Use	Wtd. Mean, % Maximum Use
D_2	Formulas (B)	4	.00014	.00019
D_3	Formulas (B)	4	.00000	.00005

Table 97. Annual Poundage Data (4190)

Substance	Number Firms Reporting 1960	1970	Poundage Reported (Matching Reports) 1960	1970	Total 1970 Reported Poundage
D_2	21	26	53,100	172,742	173,744
D_3	10	10	46,350	46,793	46,793

Table 98. Possible Daily Intakes (1851)

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
VITAMIN D2 NAS 0245	01 BAKED GOODS(R)	*	0-5 MO.	.000340	.003400	.003400
			6-11 MO.	.002540	.005180	.025400
			12-23 MO.	.005450	.008980	.034500
			2-65+ YR.	.013770	.020300	.131200
VITAMIN D2 NAS 0245	02 BREAD CERLS(R)	8	0-5 MO.	.000120	.000340	[illegible]
			6-11 MO.	.004460	.011920	[illegible]
			12-23 MO.	.005270	.010180	[illegible]
			2-65+ YR.	.004200	[illegible]	.025000
VITAMIN D2 NAS 0245	03 OTHER GRAIN(R)	*	0-5 MO.	[illegible]	.000170	[illegible]
			6-11 MO.	.000970	.002060	.009700
			12-23 MO.	.001040	.005790	.010400
			2-65+ YR.	.002740	.006140	.027000
VITAMIN D2 NAS 0245	04 FATS OILS(R)	4	0-5 MO.	.001100	[illegible]	[illegible]
			6-11 MO.	[illegible]	[illegible]	[illegible]
			12-23 MO.	.001900	[illegible]	[illegible]
			2-65+ YR.	.003500	.003320	[illegible]
VITAMIN D2 NAS 0245	05 MILK PRODS(R)	14	0-5 MO.	.001400	.004000	.005400
			6-11 MO.	.002400	.300100	[illegible]
			12-23 MO.	.004500	.174000	.054500
			2-65+ YR.	.035500	.120400	[illegible]
VITAMIN D2 NAS 0245	10 MEAT PRODS(R)	*	0-5 MO.	.000110	.001200	.000110
			6-11 MO.	.002070	.005590	.002070
			12-23 MO.	.003820	[illegible]	[illegible]
			2-65+ YR.	.007840	.013610	[illegible]
VITAMIN D2 NAS 0245	11 POULTRY(R)	*	0-5 MO.	.000050	.000230	.000050
			6-11 MO.	.000390	.001320	.000390
			12-23 MO.	.000660	.001640	.000660
			2-65+ YR.	.001290	.003230	.001290
VITAMIN D2 NAS 0245	19 SWEET SAUCE(R)	*	0-5 MO.	.006000	[illegible]	[illegible]
			6-11 MO.	.018000	.052000	[illegible]
			12-23 MO.	.052000	.152000	.052000
			2-65+ YR.	.136000	.358000	.136000
VITAMIN D2 NAS 0245	23 BEV TYPE 3(R)	*	0-5 MO.	.000240	.000360	[illegible]
			6-11 MO.	.002770	.007770	[illegible]
			12-23 MO.	.005420	.010250	.005420
			2-65+ YR.	.010400	.027770	.010400
VITAMIN D2 NAS 0245	24 IMIT DAIRY(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	.000000	.000000	.000000
			12-23 MO.	.000000	.000000	[illegible]
			2-65+ YR.	.000000	.000000	[illegible]
VITAMIN D2 NAS 0245	83 FORMULAS(B)	4	0-5 MO.	[illegible]	[illegible]	[illegible]
			6-11 MO.	.057760	.456260	.125960
			12-23 MO.	.030800	.066690	.041600
VITAMIN D2 NAS 0245	ALL CATEGORIES	23	0-5 MO.	.420390	[illegible]	[illegible]
	**************		6-11 MO.	.129420	[illegible]	.273150
	**************		12-23 MO.	[illegible]	[illegible]	[illegible]
	**************		2-65+ YR.	.219030	.565260	.380530
VITAMIN D3 NAS 0246	04 FATS OILS(R)	*	0-5 MO.	.000500	[illegible]	.000100
			6-11 MO.	.002800	.007500	[illegible]
			12-23 MO.	.002400	[illegible]	[illegible]
			2-65+ YR.	.017500	.041500	.017500
VITAMIN D3 NAS 0246	05 MILK PRODS(R)	5	0-5 MO.	[illegible]	.013400	[illegible]
			6-11 MO.	[illegible]	1.386400	[illegible]
			12-23 MO.	[illegible]	.603240	[illegible]
			2-65+ YR.	.181700	.394740	.181700
VITAMIN D3 NAS 0246	83 FORMULAS(B)	4	0-5 MO.	.000000	.000600	.167800
			6-11 MO.	.000000	[illegible]	.596200
			12-23 MO.	.000000	.000000	[illegible]
VITAMIN D3 NAS 0246	ALL CATEGORIES	10	0-5 MO.	[illegible]	[illegible]	[illegible]
	**************		6-11 MO.	.255240	1.327500	[illegible]
	**************		12-23 MO.	.257000	.614240	.266000
	**************		2-65+ YR.	.199200	.586360	.199200

Table 99. Turnover of vitamin D-milks and vitamin D-free milks at 206 convenience stores in the greater Washington, D.C., area (0903).

	% of Total Gallons		
	November 1973	November 1972	November 1971
1. Homogenized Vit. D.Milk	11.02%	15.15%	21.80%
2. Homogenized Milk 3.5% B.F.	59.24%	55.06%	61.53%
3. 2% Low Fat Milk	2.95%	2.79%	4.26%
4. 1% Low Fat Milk	18.62%	18.05%	2.83%
5. Weight Watchers (TM) Skim Milk	1.58%	1.49%	-
6. Skim Milk	4.14%	3.84%	5.27%
All other Fluid Milk & Cream	2.45%	3.62%	4.31%
Total Gallons Represented	1,029,117	902,568	824,503

1. Vitamin D fortification with 400 USP units irradiated egosterol per qt.

2. No vitamin fortification.

3. Vitamin D fortification with 400 USP units irradiated egosterol per qt. Vitamin A fortification with 2000 USP units palmitate per qt. Fortified 1.5% (by weight) Non Fat dry milk solids added.

4. No vitamin fortification. 2% (by weight) non fat dry milk solids added.

5. Vitamin D2 fortification with 400 USP units calciferol per qt. Vitamin A fortification 200 USP units of Palmitate per qt. Plus 10 mg. Ferric ammonium citrate per qt.

6. Vitamin D fortification 400 USP units irradiated egosterol per qt.

While it is premature and inappropriate to draw conclusions at this time, it may be noted that the use of Vitamin D has been associated with several therapeutic difficulties, including: individual variability of response from subject to subject; lag time between dosing and response; danger of overdose due to retention of the vitamin in tissues; and absence of a clinically convenient assay to monitor serum levels. Because of the multi-step enzymatic pathway involved in the activation of Vitamin D, it may be hoped that management of these therapeutic problems will be relieved in part through the use of 25-OHD_3. For specific indications, it may be expected that 1α-OHD_3 and 1,25-$(OH)_2D_3$ will be uniquely useful. Clinical availability of the active metabolites should provide alternative approaches to the treatment of resistant patients and eliminate the necessity of using heroic doses of Vitamin D itself with the long-term hazards involved."

C. Surveys and Research Papers

1. A paper written in 1938 by Jeans and Stearns (2983) became, and remains today, the principal scientific basis for the US RDA for D intakes. The arguments are summarized here but should be read in full in the original, because of the care with which the authors defined their data-base and the precise cautions which they attached to their inferences and suggestions.

For example (p. 703 of the paper), "The requirements of vitamin D may be defined as those amounts which, with ample intakes of calcium and phosphorus and a diet otherwise adequate, insure sufficient retention of calcium and phosphorus to permit (a) normal growth and mineralization of the skeleton and teeth of infants and children, (b) maintenance of bony and dental structures during adult life and (c) a sufficient supply for mother and infant during pregnancy and lactation. Unfortunately no one yet knows the normal rate of growth of children. Instead, there are available only many average rates of growth under widely varying conditions of nutrition."

The authors restricted their discussion to "only the ingested forms of vitamin D" as then known. Criteria of adequacy were based on "Fully adequate rather than the minimum requirement" based on x-rays of radius or tibia, supported by serum Ca and P determinations which were considered to have "definite but limited usefulness."

The authors concluded from their own studies "corroborated" by others, that infants fed cows' milk fortified with 135 IU D/quart had serum Ca and P

values that were normal (equaling those in breast-fed infants) and grew at average rates. Higher dosages (300-400 IU) produced serum values "nearer the top of the normal range" and such infants tended to grow "somewhat faster than the average." Ca retention fluctuated in children whose diet was not fortified with D. The recommendation (300-400 IU) was "tentative."

The authors stated that data were not available to show whether a level intermediate between 135 IU and 300 IU would be as effective as 300 IU. They reported, nevertheless, that amounts considerably greater than 400 IU "may be detrimental" by their criteria of growth and retention, citing 1500 IU as an example. No data on the threshold for detriment were reported, and the authors cautioned that it "may be lower than the amounts which have hitherto been administered without toxic effect."

Differences, if any, in the relative potencies in man of cod liver oil and irradiated ergosterol ("viosterol" or D_4) were small, but there was a concentration effect; probably absorption decreased in doses above 100 IU/g.

Breast-fed babies should probably receive D supplements similar to formula-fed babies, although the requirements "cannot be stated with accuracy." Such data as were available (cited) suggested that premature babies should be given 600-800 IU/day.

Few preparations of D were sold for use by children, it being assumed that they got more sunshine than infants, and criteria of need were fewer. "Prevention of rickets is of no value"; serum Ca and P were little help; no studies were known of growth rates; however, dental caries was discussed. The authors concluded that 350 or more IU/day in milk "may" lessen the incidence of caries without preventing or arresting it. However, an accurate estimate of the optimal intake of D for children could not be made on the available evidence, except that it seemed neither greater nor less than the optimum for infants.

Similarly, no estimate could be made for optimal intakes of Ca or D by adolescents. Similarly in adults; the addition of D to the diet in no way lessened the requirement for Ca. The limit of tolerance by adults appeared to be 150,000 IU or more, although the authors cautioned that the limited data showed individual variations. No conclusion could be arrived at for the needs of pregnant and lactating women, and no reports had been found of hypervitaminosis D in such women; "consequently the effect is unknown."

Nevertheless the authors concluded in their summary that 800 IU/day with an "abundant" intake of Ca and P should be given during pregnancy and lactation.

In making these recommendations in the absence of specific evidence the authors invariably prefaced their remarks with a cautionary "it would be wise" or "it would seem", after justifying a need to recommend by citing relatively adverse effects of no additions of vitamin D, and emphasizing the need for research.

2. In 1938 Nelson (4212) noted that:

(1) About one half of the evaporated milk sold was fortified with D.

(2) About 700 dairies in the US sold fresh milk fortified with D.

(3) Milk, fish, and eggs were the known natural sources of D; plants were believed not to be natural sources.

(4) The following pharmaceutical preparations were sold as sources of D:

Natural fish liver oils;
Activated sterols such as viosterol in edible vegetable oil, which contained only activated ergosterol;
Mixtures of these sources of D presented in emulsions, tablets, capsules, and malts.

The author commented that statements of minimum potency (maximum potency was not mentioned) must be relied on for intelligent use of vitamin D preparations.

3. In 1944 Johnston (2966) concluded, from a clinical study of six children aged 5-15, that D_2 intakes of 65 to 3900 IU/day were desirable and did not depress growth.

4. In 1953 Allen et al. (0064) reported the results of two nutritional surveys on 158 children aged 1-6, which included anthropometry, x-ray, biochemistry and diet records. The surveys were performed in Halifax, N.S., in 1945-1947 and 1949-1951. Observations were repeated after 6 months.

The authors commented that about half the subjects grew and calcified their bones adequately on substandard diets, but 30% did not. Bone maturation could be normal on D 25-150 IU/day and Ca and P less than 1 g; Ca 0.2-0.7 g could be adequate, but not in all cases.

5. In 1953 Baldwin (0313) reviewed the history of homogenized milks and their use as a carrier for D. Homogenization of milks dated from 1892 in France, and such milks were exhibited at the Paris World's Fair in 1900. A description of them was first published in America in 1904, and they were first marketed at

Quebec in 1910-1912, unsuccessfully. They were successfully marketed in Ontario in 1927-1932. In the United States homogenized milk was test-marketed in Illinois in 1921, and marketed commercially at Philadelphia in 1928, and in Illinois from 1932. National marketing began in 1940; by 1946 50% of milk sold was homogenized, by 1949 about 70% and by 1953 some dairies sold all their milk homogenized.

In 1932 the AMA advocated milk as a carrier of added D, and from 1932 vitamin D milks were produced by fortification, by irradiation, and by feeding cattle irradiated yeast. In 1937 they recommended general fortification of milk with D, on the ground that most people did not go to doctors unless they were ill. Dairies found that fortification was easier, and D better distributed, when milk was homogenized, and nonfat D milks became available shortly before the review was written (0313).

6. In 1957 Bongiovanni et al. (0656) found that true intakes of D in the United States were impossible to estimate. In review they noted that when the recommended daily intake in Britain was raised from 400 to 700 IU in 1943, the incidence of hypercalcemia also rose. Although, by 1957, the British fortified their milk with 1400 IU/imperial quart, compared with 400 IU/quart in the USA, many other foods were fortified with D in the USA. However, Jeans and Stearns (2993) had indicated that 800 IU/day might be excessive. From their own clinical experience the authors concluded that intakes of 400-500 IU/day should not be exceeded.

7. In 1958 Fraser and Salter (1892), from their experience and that of others, stated that they "suspect that the fortification of evaporated milk with vitamin D has had a desirable effect upon the incidence of rickets in North America", but they advise against the use of prophylactic doses in excess of 1000 IU and deprecate the unwarranted addition of vitamin D to other commercially prepared foods.

8. In 1962 Jolliffe reviewed hypervitaminosis D (0201):

a. In Britain infants had maximal intakes varying around 1500 IU/day Though intakes were less in USA, halibut liver preparations were on sale containing 6000 IU/tsp.

b. No case had been reported in a breast-fed infant, and breast-feeding was not obsolete in Britain. Human milk contained 50-100 IU D/liter, or 25% of the content of cows' milk.

c. The British Pediatric Society had recommended that no "food" fortified with D should be given to infants (who should receive cod liver oil of known potency).

9. In 1964 G. DeLuca and Cozzi (1320) reported 12 cases of hypervitaminosis D, of which 10 occurred during the spring and summer when, according to the authors, increased UV exposure decreased the need for D intake.

10. In 1966 Fraser et al. (1894) stated that in their opinion the study of Jeans and Stearns (2933) "does not stand up to modern tests of significance." They also queried the controls that associated hypercalcemia with amounts of vitamin D intakes as disclosed by British statistics; British cases were mostly mild, American mostly severe, and the causes might be different. Valid epidemiological data were lacking. The authors concluded that no good grounds existed for revising American policies on the availability of vitamin D.

11. Also in 1966 Taussig (5703) reviewed work on hypercalcemia, citing Bongiovanni et al. (0656) among others, and concluded that idiosyncracy was involved. Such children, in the author's view, were injured by D at 3000 IU/day but not at 400 IU/day; this gave only an 8-fold margin of safety. The author suggested that physicians should avoid giving children levels of D that were unnecessary and might be harmful.

12. In 1968 Stearns (5504) reviewed 25 years of her own and others' studies on vitamin D requirements, based on prevention of rickets (minimal requirements) and provision for growth of children (maximal requirements).

She concluded that:

a. Vitamin D 60-100 IU in milk was enough (daily) to prevent rickets but not enough for maximal growth.

b. A daily dose of 300-400 IU was ample for growth.

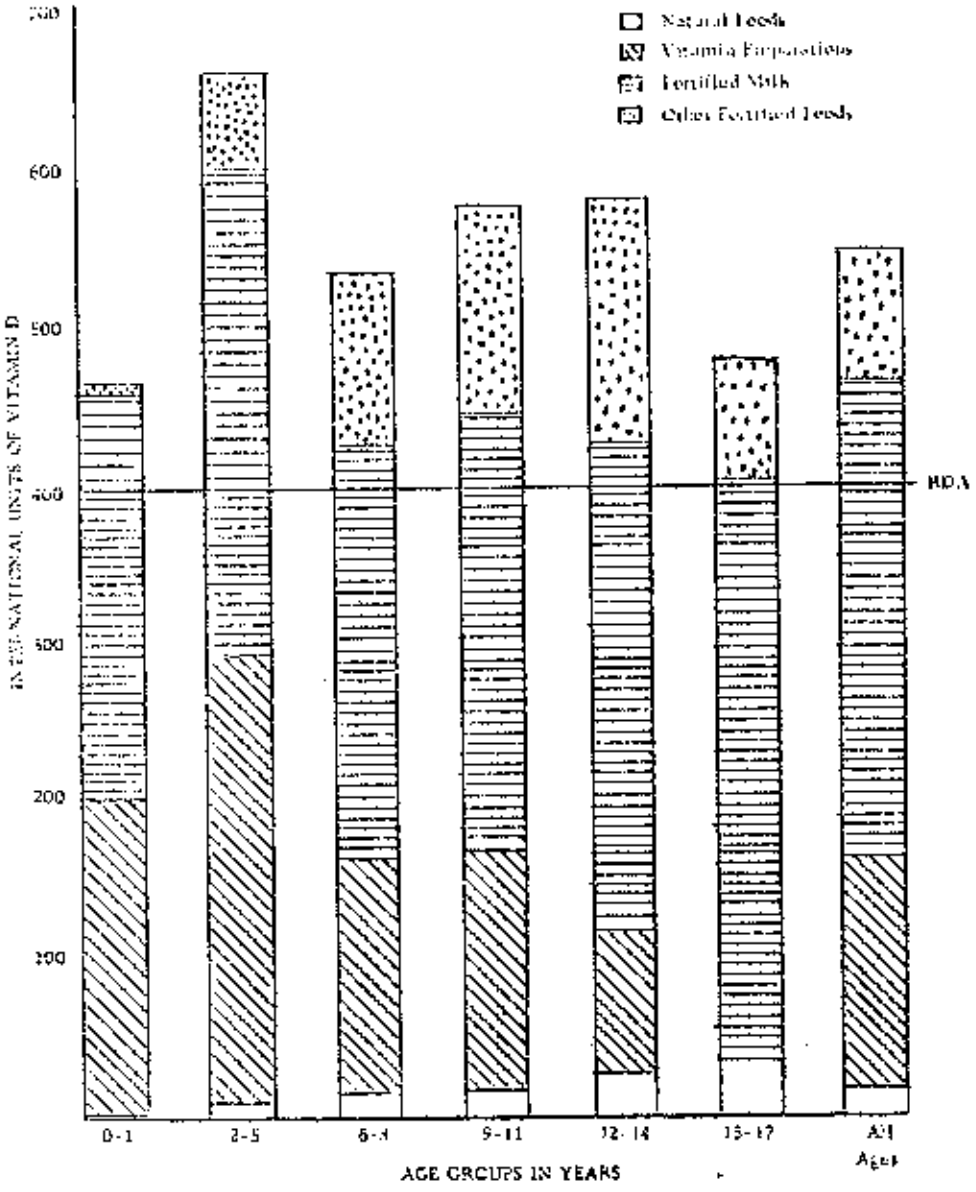
c. Growth was impeded at 2000-3000 IU daily.

She commented that it seemed "most unwise" to give children routinely 3-4 times the optimal dosage, adding that breakdown and excretion were slow, toxic levels accumulated easily, and "at present far more babies in America are being overdosed with both vitamins A and D than those receiving inadequate intakes."

13. In 1967 Dale and Lowenberg (1267) surveyed the D intakes of six groups of 25 subjects from neonates to 17-year-olds, because of reports of hypervitaminosis D. Their findings are shown in Fig. 15 and they also found that excessive intakes resulted from ingestion of vitamin preparations in addition to D-fortified foods.

Figure 15

Average daily intakes of vitamin D by age groups and sources. (1267)



14. In 1968 Cooke (1132) reviewed the etiology and frequency of infantile hypercalcemia, and concluded that:

a. The more D consumed by mother or infant, the more frequently hypercalcemia appeared. This was consistent with a normal range of hypersensitivities in a population.

b. To minimise the incidence, intakes of D and Ca by pregnant women should be limited to the RDA, and the detailed proposals by the AAP Committee on Nutrition should be put into effect.

c. These included making D-free milk available, discontinuing the addition of D to all other foods, limiting the potency of D in patent medicines, and supervising people's consumption of D, especially consumption by pregnant women and infants, and more so in families where hypercalcemia had already appeared.

15. In 1968 Kovacs (3271) wrote to C & E News on behalf of Vitamins Inc. to protest an editorial statement that vitamin D would not itself cure rickets. This statement was comment on discovery of the role of 25-OH-D_3. The author stated that D was defined officially by its effects, not by its form, and that "grossly incorrect" summaries might mislead "the common person" and so "cast aspersions" on what the vitamin industry had been doing "to make vitamin D preparations available." The author complained that "the industry is plagued by enough misinformation and attempts to regulate us" and that "the editorial staff of C & E News is misled so easily."

16. In 1969 Seelig (5216) reviewed evidence that in infants hypersensitivity to D was recognized as SAS, general arteriosclerosis, or renal acidosis. For such infants the RDA for D could be toxic; the range of sensitivities was wide, and sometimes little above the intakes that prevented rickets. White children needed less D than Black, and were more often sensitive to slight excesses. The potency of D in milk was 3-10 times its potency in oil. The author concluded that since it was difficult to guard individuals against potentially harmful intakes of D, its routine addition to foods including milk should be reconsidered. She added that specific supplements could be prescribed for children susceptible to rickets.

17. In 1970 Seelig (5217) inquired whether American children were excessively exposed to D, and pointed to risks of such exposure to hyperreactive individuals. She reviewed reports that:

a. D_2 was more toxic to experimental animals than D_3, judged by renal and cardiovascular calcification.

b. Potency of D in milk, the usual carrier, was far greater than in oil, the reference carrier.

c. Prophylactic supplements, in milk, of 125 IU/qt or 95 IU/day had been found adequate.

d. Even hyporeactive children, chiefly among ethnic minorities with deep skin pigmentation, had been protected by only 332 IU/day in milk, equivalent to 1450 IU in oil.

e. Vitamin supplements were available for hyporeactive children.

f. Much of the need for prophylaxis was seasonal.

g. The amounts needed for prophylaxis in dark-skinned children might provoke hypercalcemia in fair-skinned children, including the amounts currently added to milks.

h. As a steroid hormone, the activity of D could be increased or on the other hand abolished by minor alterations in chemical structure.

i. By the time hypercalcemia was diagnosed, brain and cardiovascular damage could be irreversible, whereas rickets could be diagnosed before irreversible damage was done.

She concluded that the level of 400 IU/qt of milk as a universal fortification reflected an "editorial compromise," and should be re-evaluated, along with methods for diagnosing D-deficiency in early infancy.

18. In 1971 Lewin et al. (3500) concluded that infants too small to consume the usual amounts of milk formula received too little exposure to D.

19. In 1971 Lumb et al. (3607) investigated the relationship between serum D values and exposures to D, during a study of normal subjects and patients with D-resistant kidney-related Ca disorders. Some cases received therapy with D_2 or D_3.

Healthy serum D values were found to range between 0 and 1.6 IU/ml, average 0.77. The authors commented that:

a. normal or least-sufficient values were unknown,

b. the conventional 1 IU/ml was arbitrary, and

c. values varied among populations; in North America there was more sunlight than in Britain, also more exposure to D supplements.

The authors noted that 75 IU/day probably was enough for health of a human adult; cases had responded to 100 IU/day, and to hospital diets without D supplements. However, they concluded that casual exposure to sunlight was not enough.

They cited a survey indicating that British intakes of D seldom exceeded 150 IU/day, but claimed to have found no systematic survey of the US population. Nevertheless they concluded that differences of D-related clinical observations probably reflected differences of exposure to D. Serum Ca of healthy subjects showed no such differences.

20. In 1972 Seelig (5219) reviewed her own and others' evidence that high intakes of D increased urinary losses of Mg and thus increased the Mg requirements of infants. There was a connection between hypomagnesemia and hypercalcemia in some patients. Although much of the evidence was indirect, or from animal studies, Seelig concluded that D-supplemented cow's milk imposed a greater Mg requirement in infancy than did mother's milk, and that long-term studies were urgently needed.

21. In 1973 Palmisano (4431) surveyed the exposure to vitamin D, and the attitude of physicians to it. He drew attention to the following:

a. Adult skin contained 3-4% of 7-dehydrocholesterol (7DHC) under the stratum corneum; infants more than double, and their corneum was thinner and less pigmented, regardless of race.

b. Most commercial milks, baby foods, and breakfast cereals were fortified with D. When added to endogenous D, average Americans might receive several times their RDA. Some Americans showed profound toxicity to amounts only slightly over their RDA. In normal persons intakes over 100,000 IU/day were toxic (symptoms listed) and such persons were liable to metastatic kidney calcification and hypercholesterolemia.

c. D-deficiency in the absence of malabsorption was rare; total intakes above 400 IU/day from all sources (author's emphasis) were not required, and intermittent exposure to sunlight would provide this except to strict vegetarian recluses. Minimum requirements had been estimated as 70 IU/day, except that dark skinned persons converted 7DHC to D_3 less efficiently than light skinned persons.

22. In 1973 a Lancet Editorial (0193) discussed the need for vitamin D supplements. Rickets had been eliminated in Britain after addition of D_3 to National Dried Milk, but had reappeared since 1963, and was estimated to occur in 4% of school children. UV deficiency was estimated to be common in senior citizens, and to contribute to osteoporosis. A Panel on Nutrition of the Elderly had recommended fortifying their liquid milk.

23. In 1973 an editorial in the Journal of the American Medical Association (1631) also stated that 1,25-$(OH)_2$-D_3 acted "in the manner of a true hormone", that it had therapeutic uses to be studied, and that in research its use might uncover the etiology of some hitherto baffling disorders.

D. Recommendations by Industrial Organizations

1. In 1973, the National Dairy Council (4191) commented on the reaffirmed (1968) RDA of Ca, 0.8 g/day, as "expressed without reference to" other dietary intakes affecting Ca utilization, as to which the keynote was uncertainty:

a. Presence and roles of the 1 or 2 CaBP's of human intestine,

b. Geographical differences and biochemical influences on real availability of vitamin D,

c. Intakes of P, and Ca:P ratio, recommended as 1:1 but currently estimated as 1:2.8, which influence Ca utilization,

d. Dietary protein influences on uptake and retention of Ca,

e. Enhancement of Ca uptake by dietary lactose,

f. Influences of dietary Mg; fats, bile, bile salts; oxalic and phytic acids in vegetables and cereals.

Compared with the United States 0.8 g/day, the RDA of Ca was 0.5 g/day in Canada and Britain and 0.4–0.5 g/day was recommended by FAO/WHO. Methods of estimating the RDA were diverse, and few studies had allowed for adaptation time. Many studies were based on assumptions not backed by evidence, or on claims that deficiency diseases had not been observed. Neither the risks nor the requirements had been established, and it was possible that the RDA of Ca should be increased above 0.8 g/day (4191).

2. In 1968, Brooke (0770) noted that although D and Ca were optional ingredients in the Standards of Identity for Enriched Flour and Bread, little interest had been shown in adding them at the mill, but bakers started adding them for a short while in 1953. However, the practice was discontinued (see also 0079, 0771).

3. In July 1954 the American Institute of Baking (0079) advised the industry to "refrain from using its prerogative to add vitamin D to bread" or, if they did so, not to add more than 400 USP units/lb and then to add enough Ca "to permit simultaneous claims for calcium." The Institute cited a statement by the NAS NRC that it was "unnecessary" to add D to bread, and added that such addition "contributes little to the nutritional welfare of the American public."

4. However, according to Brooke (0771), the response by members of the American Institute of Baking to the circular of July 1954 seems to have been incomplete; also, many pointed out that the urban poor could not afford to pay the extra penny that was then charged for D-enriched milk.

E. Recommendations by Professional Bodies

1. In 1963 the Committee on Nutrition of the American Academy of Pediatrics (1118) issued a 14-page statement of policy on requirements and toxicity of vitamin D. In their opinion:

a. "Intakes of 250 IU daily are at least as effective as greater intakes".

b. Requirements of Negro infants did not differ from those of White infants.

c. Use of massive single doses (300,000 IU) was unphysiologic and unnecessary.

d. Premature infants should receive D supplements in the first 2 weeks of life, but did not need more than 100–200 IU/day.

e. Minimal needs of older children, adolescents, adults, and pregnant and lactating women could not be defined for lack of evidence.

f. Toxicity findings were "contradictory" but intakes considerably lower than 3000–4000 IU could "sometimes" be toxic.

g. Exposures in USA or Canada "might reasonably exceed" 2000 or 3500 IU/day and enrichment of foods other than milk could not be justified. The long term consequences of such intakes were unknown.

The Committee recommended that total daily intakes of infants and children should be maintained at 400 IU and should be restricted to this amount from all sources. They warned of the variety of sources. To implement this recommendation they concluded that "commercial vitamin D supplements should be adjusted to contain not more than 400 IU per dose" (sic).

2. In 1965, the same AAP Committee (1119) issued an addendum with copy to the FDA, stating:

a. Because hypercalcemia (severe) was now reported in utero, the 400 IU/day limit should apply to pregnant women as well as infants.

b. Addition of D to foods other than milk and infant foods should cease.

c. Products containing more than 400 IU/dose should be sold only by prescription.

d. All products containing D should be labeled with a warning of possible toxicity if taken in excess of the stated dose.

e. All doctors should establish a specific need by tests before prescribing D. "Normal adults living under ordinary conditions do not require supplementary vitamin D."

3. In 1967, the same Committee issued a third statement (1893) again emphasizing that D was "potentially dangerous in very high dosage" and that the margin of safety had not been established. The report was largely concerned with re-reviewing hypercalcemia; it stated that 400 IU/day was ample for mother and fetus normally, that practically nothing was known of the minimum requirements, and that the metabolism of mothers of severe hypocalcemics had not been studied. Laboratory findings in severe hypocalcemics were inconsistent, for unknown reasons, and though it had been surmised that such patients were hypersensitive to D, loading tests that would verify such suggestions had not been reported.

In Britain the incidence of severe cases had been estimated as 1:275,000 live births, and in Toronto as 1:120,000 live births, but the basis for these estimates could be criticized.

Prophylactic requirements in infants could be as little as 100 IU/day, and for older children 400 IU/day was "considerably more" than the minimum. There was no reason for normal persons to consume more than this; equally, there was no reason to compel public health authorities to insist on less.

The earlier recommendations were repeated:

a. Milk to be enriched with 400 IU/qt.

b. No enrichment of any other foods.

c. All D supplements to contain 400 IU/recommended dose/day.

4. In addition they (1116) drew attention to the lack of vitamin D in nonfat dry milk, fed increasingly to infants. They stated that the lack of fortification was because D had not been included by Congress in the 1944 Standard of Identity for nonfat dry milk, and recommended that D supplements be given to infants receiving it.

5. In 1967 the AAP Committee on Nutrition (1117) also commented on some regulatory changes proposed by the FDA. They reiterated an earlier recommendation that:

a. 400 USP units/day be the intake of infants.

b. 250 USP units/day be the minimum intake supplied.

c. A level of 40 USP units/100 kcal of formula would be "appropriate".

6. In 1968 the Food and Nutrition Board of the NAS NRC (1849) reissued their RDA for vitamin D. They recommended 400 IU/day separately for infants, children, males up to age 22, females up to age 22, pregnant women, and lactating women. No recommendations were given for males or females above age 22. No intake level other than 400 IU/day was recommended for any category.

In explanation the Board stated:

a. D was essential at all ages, was acquired by exposure to UV, or by ingesting D_2 or D_3, and became deficient when the total from all sources was inadequate.

b. 100 IU/day had prevented rickets in normal infants, and 100-200 IU/day had been enough to prevent rickets in premature infants; 300 IU/day had cured "actively" rachitic infants.

c. Although rickets beyond infancy was "virtually unknown" in North America, and requirements for D were "difficult to determine beyond infancy", the Board "reaffirmed" an RDA of 400 IU/day for children and adolescents.

d. Although the adult requirement for D "is not known", the Board stated that 400 IU/day was "also justified for older children and adults". No references were cited for this statement, although references were cited for the other recommendations mentioned. Furthermore, night-workers and nuns were advised to drink vitamin D milk (no references cited, and no other occupations mentioned).

e. At the same time, intakes "considerably less than" 2000-3000 IU/day had been toxic to some people, and "the long-range effects of small excesses of vitamin D have not been extensively studied in vitamin D-sensitive individuals".

f. Actual individual intakes were difficult to assess because many foods were fortified with various, and varying, amounts of D. Thus many people "considerably exceeded" the RDA. Furthermore, the RDA was ingested without supplements by most people of all ages, except by infants fed breast milk alone or unfortified formulas.

7. In 1970, a Joint WHO/FAO Expert Group (2969) reported that D was supplied both by animal foods and by sunlight, so that amounts available were difficult to estimate. In the tropics, intakes were low but there was ample sunlight. In temperate areas, intakes needed to be supplemented. However, intake data were "virtually non-existent", except for a British estimate of 116-133 IU/person/day covering both rural and urban populations.

The Group stated that RDA criteria were normally derived from:

a. Non-deficient populations surveyed,

b. Deficient populations surveyed,

c. Controlled human experiments, the production, prevention, and cure of deficiency; these were the most valuable, and

d. Animal experiments.

When there was agreement among the above sorts of data, the RDA could be estimated. With D, the sunlight factor contributed uncertainties. Furthermore, the Group emphasized that RDA for ingestion of nutrients in foods were not intended to be the sole bases for evaluating the nutritional status, especially of individuals.

The Group cited the Jeans and Stearns paper (2933) as still valid, and felt that the upper limits of their recommended ranges of intakes should be used. They noted, at the same time, that studies on older children and adults were still lacking; however, they discussed four reports that patients with osteomalacia had responded to 100 IU/day. They remarked that the amounts of exposures to sunlight had not been measured, and remained unknown. Apart from sunlight, there was no evidence that D requirements were influenced by climate, altitude, temperature, body-weight, excercise, or sex.

In the absence of significant information on the availability of D from foods, the Group "assumes" that all D in all foods is 100% absorbed. They considered toxicity to be a hazard of intakes much greater than those recommended below, citing hypercalcemia as a result of long exposure to intakes of 3000-4000 IU/day in infants.

The Group recommended the following intakes of D:

Birth to age 6	10 μg (400 IU) daily
Age 7 and above	2.5 μg (100 IU) daily
Second and third trimesters of pregnancy	10 μg (400 IU) daily
Lactation	10 μg (400 IU) daily

8. In a further report of 1970 the Committee (1844) repeated that there was abundant evidence that fortification of milk and infant foods were effective against rickets. A survey in North Africa had revealed 45-60% of hospitalized infants as rachitic, and preventive programs in developed countries were not fully effective. However, in Britain when the D level in National Dried Milk (given out at Government postnatal clinics) was raised from 10 IU/g to 18 IU/g there had been an increased incidence of hypercalcemia. After the level was reduced to 3.2-3.5 IU/g and the levels in infant cereals had been reduced from 35 to 10 IU/g and in cod liver oil from 800 to 400 IU/tsp, the incidence of hypercalcemia returned to "low levels". Nevertheless, infants could still get 1000-1200 IU/day.

The Committee noted that in Canada D was added only to milk and margarine, at closely controlled levels and that the American Academy of Pediatrics had recommended that only milk and infant formulas be fortified in the United States so as to maximize intakes at 400 IU/day/infant.

The Committee emphasized the apparently large differences in individual sensitivity to vitamin D. They cited opinions that congenital supravalvular aortic stenosis might reflect hypervitaminosis D. They concluded that current programs were "not entirely satisfactory" either for preventing rickets or for preventing hypervitaminosis D.

9. In 1973 the Food and Nutrition Board of the NAS NRC (1848) issued a policy statement on improvement of the nutritional quality of foods, announced as superseding their statement of 1968. After saying that nutrients for which RDA were specified were provided in adequate amounts by a properly selected diet, they "endorsed" the enrichment, fortification, and restoration of the nutritional values of certain foods, including "the addition of vitamin D to milk, fluid skim milk, and nonfat dry milk."

F. Regulatory Status

United States

1. In 1965 the FDA published a proposal for rule-making (0155, 1845) over concern that excessive intakes of vitamin D might cause infantile hypercalcemia.

The rules were intended to have the following effects:

a. D would be permitted in "food supplements supplying not more than 400 USP units per day."

b. "Vitamin D preparations containing over 400 USP units per day" would be sold only on prescription.

c. Any drug containing doses of more than 400 USP units/day and sold over the counter would be classed as "misbranded."

d. Preparations supplying less than 400 units/day would be "misbranded," because the layman is "not qualified to diagnose or treat" D deficiencies.

2. In 1966 the US Department of HEW (5876) issued a publication explaining Grade A milk to the public, and affirming that milk described as "vitamin D milk" contained 400 IU/qt, or about 100 IU/glass.

3. In 1968 the 1965 FDA proposal was withdrawn (0172) because of long-drawn-out controversy over it.

4. In 1971 the FDA announced (0188) that capsules containing 50,000 units of D_2 would be regarded as "new drugs" subject to NDA (new drug applications for approval) or revised applications if already approved. They added that D_2 lacked "substantial evidence of effectiveness for use in lupus vulgaris." Such high doses of D_2 must be labeled "Caution: Federal law prohibits dispensing without prescription" and, under "Indications", "For use in the treatment of hypoparathyroidism and refractory rickets."

5. In December 1972 the FDA acted to limit the potency of over-the-counter preparations A and D (1846), the Commissioner commenting:

> "Vitamins A and D are known to be toxic; and they are heavily promoted in high doses to the consumer; and we continue to accumulate evidence of adverse effects from excessive intake. The FDA has concluded, therefore, that consumer safety requires the action we are taking."

Daily limits of 400 units (upper and lower) were proposed for D, and the proposal noted that many preparations on the market contained less, and some "60 times the RDA," and that neither A nor D was proven effective for conditions such as acne, night-blindness, and arthritis in well-nourished people. Sixty days were allowed for comment.

6. In July 1973 the FDA announced that "International Units" would replace the term "U.S.P. Units" to describe the potency of D, giving September 1, 1974, as the last date for comments on this proposal (0196).

7. In March, 1973, the FDA issued regulations for nutrient labeling, that covered additions of vitamin D, to take effect on January 1, 1974 (0198). Major provisions included:

a. If a vitamin, mineral, or protein was added to a food, it was covered by the regulations, and it was also covered if nothing was added but the label contained a specific claim of nutritional value.

b. Additional rules applied if the labeled food provided 50% or more of the RDA of any regulated nutrient.

c. The U.S. RDA for vitamin D was reconfirmed as 400 IU/day.

d. If a food contained 10% or more of the U.S. RDA it could be labeled as a "significant source" of the vitamin. A food could be claimed as "superior" to another food if the superiority amounted to 10% or more of the U.S. RDA for the relevant nutrient.

e. Added nutrients were designated as Class I, nutrients naturally present were designated as Class II. Foods were "misbranded" if their contents of Class I nutrients were less than all of those claimed on the label, or less than 80% in the case of Class II nutrients.

These regulations represented action on a proposal published on January 19, 1973, asking for comments. The Commissioner of Foods and Drugs reviewed (0198) the comments and his conclusions at length (see original document). One of his conclusions was that a product with nutrient(s) added so that the product supplied 50% or more of the U.S. RDA "is properly regarded as a dietary supplement rather than a food," which did not apply to foods naturally containing more than 50% of the U.S. RDA and sold without additions. These measurements were per serving, as defined in the regulations.

8. In August, 1973, the FDA further defined the above labeling requirements, with permission to alter labels accordingly from August 2, 1973, and with enforcement after December 31, 1974 (to include any further rules issued during 1974) (0197). These definitions established standards of identity for "food for special dietary uses", including vitamin D supplements.

a. The following U.S. RDA were defined (Table 100).

b. The U.S. RDA are in fact derived from the RDA recommended by the NAS NRC, with which they are identical. But the term "U.S. RDA" is distinct, and applies only to those RDA that have been affirmed by the FDA in legislative action.

c. Basically, all preparations containing D are "foods". However, if a single serving or other dose-unit (e.g., vitamin pill) contains 50% or more of the U.S. RDA, it must be called a "dietary supplement". This regulation also applies to products containing less D than 50% of the U.S. RDA if claims are made that they can be used to supplement the daily diet with essential nutrient(s). It also applies to products that are single vitamins or minerals.

Table 100. U.S. RDA for Vitamin D, Calcium, and Phosphorus (0197)

	Children under 4 years			Adults and Children of 4 years and over			Pregnant or Lactating Women		
	Limits			Limits			Limits		
	lower		upper	lower		upper	lower		upper
Vitamin D(IU):									
mandatory	200	400	400				400		400
optional				200	400	400			
Calcium (mg):									
mandatory	125	800	1200	125	1000	1500	125	1300	2000
Phosphorus (mg):									
mandatory	125	800	1200	125	1000	1500			
optional*							125	1300	2000

*Must be no greater than the amount of calcium.
Mandatory means that it must be included in multivitamin preparations.
Optional means that it need not be included.

d. If a preparation is intended for use by special consumer groups:

"Infants

Children under 4 years of age

Adults and children 4 or more years of age

Pregnant or lactating women"

the label shall say so, and, if more than one group is mentioned, it shall specify the daily amounts recommended for each group separately.

e. The final, total amount of D present in foods to which D has been added, and not just the amount added, is the subject of these regulations. There is an exemption for foods in which the natural content of D, present before processing, has been no more than "restored", provided that the restoration is complete. There is also an exemption, in part for foods naturally containing more than 50% of the U.S. RDA per serving or dose-unit, provided they are described as "dietary supplements".

f. Other labeling requirements include expiration dates, and both content and typography of the label. See original Regulation.

g. If, in the case of D, a single serving or dose-unit contains more than 100% of the U.S RDA, it is classed as a prescription-only drug. There is one exemption from this. Foods represented for use "solely under medical supervision to meet nutritional requirements of persons with poor vitamin D absorption" may contain up to 1000 IU/dosage unit or recommended daily intake. Such foods may be bought freely without prescription.

h. The regulations do not otherwise restrict over-the-counter sales of D. Nor do the Regulations mention the GRAS List.

Canada

The regulations are given in official sources (0202) and some key points are noted in a transmittal letter from the Canadian Department of Health and Welfare (4084).

(1) Vitamin D, D_2, or D_3 may be added only to margarine and other butter substitutes, to prepared infant formulas, or to four categories of milk and milk products.

(2) The amounts that may be added are specified by reference to a table of Reasonable Daily Intakes (RDI).

(3) Foods without added vitamin D may be advertized as "excellent" sources of it if the RDI would contribute 300 IU/day or more.

(4) A food with added vitamin D may not be offered for sale if its RDI would contribute less than 300 or more than 400 IU/day; separate regulations are made for adults and for children under two. Any food with added vitamin D must be labeled with the amount added, as IU/100 g or 100 ml.

(5) Any dose-form that would contribute more than 400 IU/day is classed as a drug. If it contributes no more than 1000 IU/day it can be sold to the public but must not be advertized, and the container must be labeled for therapeutic use only. If it contributes more than 1000 IU/day it must be prescribed. If it contains less than 200 IU/day it cannot be sold as a drug.

(6) These rules apply also to combination drugs containing vitamin D as one component: for children under six the recommended daily dose must supply at least 200 IU, and labeling and advertizing requirements are similar to those governing vitamin D additions to foods.

(7) "Drug" doses of vitamin D can be sold (without prescription) only to drug manufacturers, wholesale druggists, registered medical practitioners or pharmacists, hospitals, or Government Departments. Special labeling is required. The manner in which vitamin D may be sold as a prescription drug is also regulated.

(8) An advertisement or a label may claim or imply that the presence of a lawful amount of vitamin D is a factor in the normal development and maintenance of bones, teeth, and good health, especially in infancy and childhood. Apart from specifying the amounts added, no other claim of any sort may be made or implied for vitamin D.

United Kingdom

The regulations have been much amended and are currently being revised (1732).

(1) Vitamin D is defined as the "anti-rachitic vitamins" (0165) including D, D_2 (ergocalciferol), and D_3 (cholecalciferol) and is currently measured in terms of IU or micrograms of cholecalciferol; after December 31, 1974, IU will no longer be used (0190).

(2) When vitamin D is added to foods the minimum amounts added must be shown on the label: maximum penalties are 3 months in prison and/or a $240 fine plus $12.50 (approximately) for each further day of noncompliance (0183).

(3) The only foods fortified by law seem to be National Dried Milk (supplied from Government clinics and amounting to about 0.25% of milk products consumed) and margarine which accounts for 35-40% of UK vitamin D intakes (1732). All margarine sold must contain between 80 and 100 IU vitamin D/ounce (0165).

(4) Baby foods, and condensed and other milks, and "many other items" but not flour or bread, are stated to be fortified electively with vitamin D (1732). Milks account for 8-10% of UK intakes of vitamin D (1732).

(5) Fish is a source of natural vitamin D, accounting for 18-20% of UK intakes (1732).

(6) If a "medicinal product food" is not labeled with dosage directions, or if an adult dosage is recommended that includes more than 250 "units of antirachitic activity" per day, the product can be sold only under the provisions of a licensing system (0185).

(7) In 1973, it was recommended that medicinal products containing over 10 micrograms of cholecaliferol (400 IU) should not be "on general sale", and those on sale should carry a warning that only half-doses should be given to children receiving dried milk (0199).

Netherlands

The following information was supplied on February 21, 1974, by the Royal Netherlands Government in response to an inquiry (4450):

> "In the Netherlands the addition of vitamins to foods is in general forbidden. In special cases, where the need for and usefulness of supplementation have been clearly proven, the Minister of Health will designate suitable vehicles for obligatory supplementation (Food Law, General Decree, Art. 10 bis).
> In view of eating habits and climatic conditions in the Netherlands the need for extra vitamin D has been accepted as proven.
> As a vehicle for supplementation "margarin" (an edible water in oil emulsion with 80% fat) has been designated and 3 I.U. of vitamin D3 have to be present per gram of margarin (Food Law, Margarin Decree, Art. 2, Par. 2).
> In addition "Infant formula" shall contain a minimum of 60 I.U. and a maximum of 120 I.U. of vitamin D per 100 kcal.
> Apart from these two exceptions ("margarin" and "infant formula") no additions of vitamin D in foods for sale in the Netherlands are permitted."